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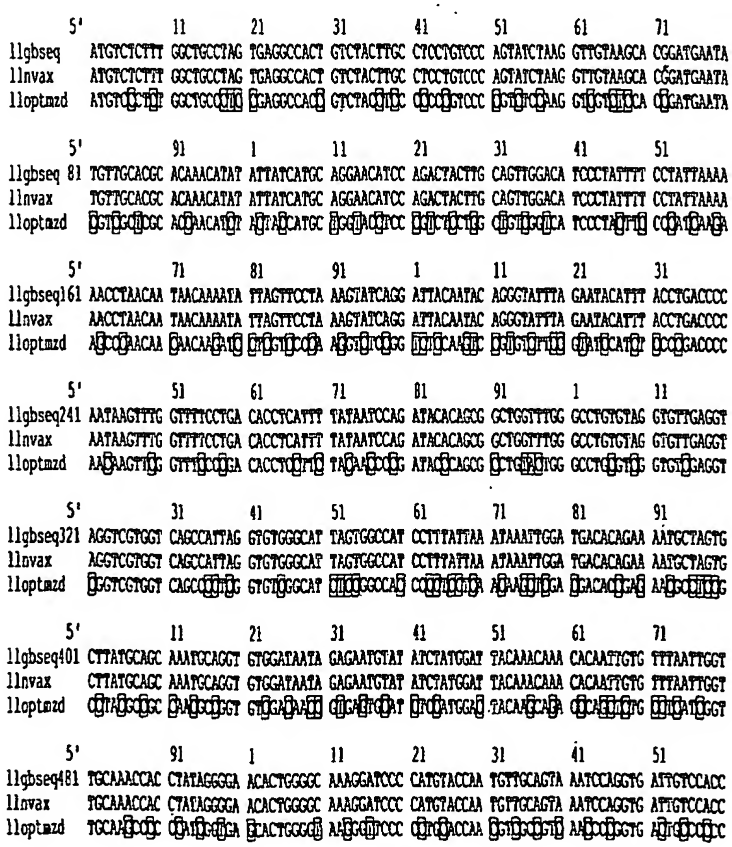
(54) Title: OPTIMIZATION OF GENE SEQUENCES OF VIRUS-LIKE PARTICLES FOR EXPRESSION IN INSECT CELLS



(57) Abstract: Codon optimized polynucleotides for optimal expression of recombinant proteins in eukaryotic cells are provided. The codon optimized polynucleotides encode a viral capsid protein that self assembles into a virus-like particle. The virus-like particle is expressed extracellularly and exhibits conformational antigenic epitopes capable of raising neutralizing antibodies. Pharmaceutical compositions, vaccines, and diagnostic test kits containing the gene products of the codon-optimized polynucleotides are also provided.

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Optimization Of Gene Sequences Of Virus-Like Particles For Expression in Insect Cells

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit under 37 U.S.C. § 119(e) based on U.S. Provisional Application Nos. 60/356,119, 60/356,161, 60/356,118, 60/356,133, 60/356,157, 60/356,156, 60/356,123, 60/356,113, 60/356,154, 60/356,135, 60/356,126, 60/356,162, 60/356,150, 60/356,151, and 60/356,152, each filed February 14, 2002, the entire contents of each of which are incorporated herein by reference.

I. FIELD OF THE INVENTION

[002] The present invention relates to the field of viral vaccines, therapeutics, and diagnostics, compositions and methods for the detection, protection and treatment of human papillomavirus (HPV) infections and associated dysplasia. In particular, the invention relates to novel polynucleotide molecules encoding recombinant HPV gene products having increased antigenicity and immunogenicity in mammals.

II. BACKGROUND OF THE INVENTION

[003] Cervical cancer results in over 200,000 deaths per year worldwide (Parkin *et al.*, 1990; Pisani *et al.*, 1990). The greatest burden of disease is in developing countries, where cervical cancer is the most frequent female malignancy and comprises 25% of all female cancers. Cervical dysplasia makes up 7% of all female cancers and causes greater than 5000 deaths per year in the U.S. (Shah and Howley, 1996). Through clinical studies, epidemiologists have identified human papillomavirus (HPV) as the major cause of cervical cancer and cervical

dysplasia. (Walboomers *et al.*, 1999). On a worldwide basis, most cervical cancers contain the genes of "high-risk" HPVs (genotypes 16, 18, 31, and 45) (Bosch *et al.*, 1995; Walboomers *et al.*, 1999). The nucleotide sequences of human and animal papillomavirus genomes are accessible in GenBank.

[004] HPV-16 is found in approximately 50% of cervical cancers, and HPV-18, HPV-31, and HPV-45 account for an additional 25-30% of HPV-positive tumors. Though early detection of HPV-induced cervical neoplasia is possible with Papanicolau (PAP) smears and cervicoscopy, screening programs in developing countries are only now emerging. In the United States, where the widespread availability of PAP screening and other methods have been associated with a reduction in the incidence of cervical cancer, the annual economic loss in the U.S. is still estimated at $5 billion (Kirnbauer *et al.*, 1993). Effective HPV vaccines would reduce the prevalence of worldwide cervical cancer and reduce the cost of screening and treating premalignant cervical disease.

[005] Prophylactic viral vaccines that efficiently prevent infection or modify disease have a successful record as cost-effective approaches to prevent and manage viral diseases. Human papillomaviruses are DNA tumor viruses that encode several viral oncogenes. Two of these viral oncogenes, E6 and E7, are conserved and expressed in human genital warts, dysplasia, and tumors, and may be required for maintenance of the tumorigenic phenotype. These features raise theoretical arguments against a HPV E6 and/or E7 subunit protein or DNA vaccine consisting of these viral proteins alone. Wild type and intact versions of these viral genes and their gene products in the context of a vaccine may disrupt normal host cell gene regulation by increasing the levels of Rb and p53 proteins and facilitate cell transformation.

Subunit protein viral vaccines utilizing virus-like particles (VLPs), analogous to the hepatitis B virus vaccine derived from yeast, have been developed as prophylactic vaccines to prevent viral infections and diseases including HPV infections (Schiller and Lowy, 1996; Cook *et al.*, 1999; Harro *et al.*, 2001).

[006] Papillomaviruses encode the major capsid gene, L1, whose gene products are able to self-assemble into virus-like particles in the absence of other viral gene products (Kirnbauer *et al.*, 1992; Hagensee and Galloway, 1993; Kirnbauer *et al.*, 1993). Recombinant papillomavirus L1 VLPs display several properties that are advantageous for vaccines. These features include the following: (1) similar size and morphology as natural papillomavirus virions as shown by electron microscopy, (2) immunodominant and conformational epitopes present on natural virions as determined by immunodetection assays with neutralizing monoclonal antibodies, and (3) elicitation of high titers of type-specific neutralizing antibodies as seen in sera of vaccines (Kirnbauer *et al.*, 1992; 1993).

[007] Several trials of preventive papillomavirus vaccine candidates using L1 VLPs purified from insect cells have been conducted in animals using the cutaneous cottontail rabbit papillomavirus (CRPV) disease model, the oral mucosal bovine papillomavirus 4 (BPV4), and canine oral papillomavirus (COPV) models in cattle and dogs, respectively. Three subcutaneous injections of CRPV L1 VLPs given without adjuvant, or combined with alum or Freund's adjuvant, protected rabbits for at least one year against persistent infection and subsequent carcinoma after high-dose CRPV challenge (Breitburd *et al.*, 1993; Christensen *et al.*, 1996). Similarly, calves and dogs given two intramuscular injections of BPV4 L1 VLPs (with alum) and COPV L1 VLPs (without adjuvant), respectively, were protected from subsequent oral mucosal

challenge (Suzich *et al.*, 1995; Kirnbauer *et al.*, 1996). In the CRPV and COPV models, passive transfer of serum or IgG from animals immunized with the L1 VLPs protected animals challenged with the homologous virus, indicating that neutralizing antibodies were sufficient to confer protection (Breitburd *et al.*, 1993; Suzich *et al.*, 1995).

[008] Recombinant peptides or proteins encoded and expressed by custom synthesized genes often require further modifications. These peptides have often lost their ability to fold and show no disulfide bond formation. Thus proteins frequently are not stable in the presence of endogenous bacterial proteases, and tend to aggregate into inactive complexes. Consequently, recombinant peptides often suffer from low yield and demonstrate reduced antigencity and immunogencity as compared with native peptides.

[009] Purification of heterologous recombinant proteins from baculovirus-infected insect cells demonstrated that host contaminant proteins were best separated from the recombinant protein using an ion exchange step as the first step in the protocol (Robinson *et al.*, 1998). Purification of baculovirus-derived HPV L1 VLPs (Kirnbauer *et al*, 1993; Suzich *et al.*, 1995) and yeast-derived intracellular HPV L1 VLPs (Cook *et al.*, 1999) were described previously.

[0010] The invention as disclosed and described herein, overcomes the prior art problems with HPV therapies through the generation of novel synthetic polynucleotides that encode HPV capsid genes encoding HPV capsid proteins capable of assembly into VLPs. The capsid proteins of the invention retain their optimum native folding and exhibit conformational presentation of epitopes that elicit antigen-neutralizing antibodies. Large scale production and purification of

HPV-L1 VLPs and HPV chimeric VLPs and their manufacturing for vaccines and other pharmaceutical products are also disclosed.

III. SUMMARY OF THE INVENTION

[0011] This invention is directed toward the prevention, treatment, and diagnosis of papillomavirus infections and associated benign and neoplastic diseases in humans. In particular, the invention discloses novel synthetic polynucleotides capable of expressing highly immunogenic HPV VLP and HPV chimeric VLP products.

[0012] According to one aspect of the invention, there is provided a codon-optimized polynucleotide encoding a viral capsid protein that self assembles into virus-like particles exhibiting conformational antigenic epitopes capable of raising neutralizing antibodies wherein the virus-like particles are expressed from a host cell extracellularly.

[0013] The viral capsid protein is from an enveloped virus, or a non-enveloped virus. Preferably, the virus comprises rotavirus, calicivirus, hepatitis E virus, papillomavirus, influenza virus, hepatitis C virus, retrovirus, or a combination thereof. More preferably the virus is a human papillomavirus.

[0014] In one embodiment, the codon-modified polynucleotide comprises SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1.

[0015] In another aspect, the invention provides pharmaceutical compositions for treating, ameliorating, or preventing a papillomavirus related disease or disorder comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the

virus-like particles are expressed from a host cell extracellularly, and an acceptable carrier or diluent.

[0016] In one embodiment, the pharmaceutical composition comprises: (a) a polypeptide which is encoded by a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1, (b) a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1; (c) a vector carrying a polynucloetide a molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1; or (d) transfected, or generally transformed with a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1; and a pharmaceutically acceptable carrier or diluent.

[0017] In yet another aspect, the invention provides a vaccine composition to induce immunity against a papillomavirus infection in humans comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the virus-like particles are expressed from a host cell extracellularly, and an adjuvant.

[0018] The vaccine provides humoral immunity, cell-mediated immunity, or both. The vaccine protects against papillomavirus infections that are caused by one or more human papillomavirus genotypes.

[0019] In another aspect, the invention provides a diagnostic test kit for detection of papillomavirus infection comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the virus-like particles are expressed from a host cell

extracellularly, and a detection agent comprising a detectable label. Preferably, the diagnostic test kit detects papillomavirus infections that are caused by one or more human papillomavirus genotypes.

[0020] In yet another aspect, the invention provides a vector comprising the codon-optimized polynucleotides of the invention operatively linked to an eukaryotic or a prokaryotic regulatory control element, capable of replication in a prokaryotic host, eukaryotic host, or both. Transformed cells carrying the vector are also disclosed.

[0021] In yet another aspect, the invention provides a method for preparing a codon-optimized polynucleotide comprising one or more of the following steps: (a) replacing codons that are underutilized in insect cells with codons that are utilized at high levels in insect cells, to create an initially-modified nucleotide sequence; (b) modifying the initially-modified nucleotide sequence by choosing a preferred codon for the initially modified sequence, wherein: (i) the ratio of GC nucleotide pairs to AT nucleotide pairs in the further-modified nucleotide sequence trends toward approximately 1:1; (ii) the number of palindromic and stem-loop DNA structures in the further-modified nucleotide sequence is minimized; and (iii) the number of transcription and post-transcription repressor elements are minimized.

[0022] In another aspect, the invention provides a method of, treating, ameliorating, or preventing a papillomavirus-related disease or disorder comprising administering to an individual in need thereof an effective amount of the pharmaceutical composition of the invention.

IV. BRIEF DESCRIPTION OF THE FIGURES

[0023] FIGS 1A-1C show the alignment of two wild-type HPV-16 papilloma virus L1 polynucleotide sequences with a codon-optimized HPV-16 L1 polynucleotide of the invention. The aligned sequences are: HPV-16 L1 wild-type sequence from GenBank record Accession No. K0278 ("l1gbseq"; SEQ ID No. 12), HPV-16 wild type clone NVAX ("l1nvax"; SEQ ID No. 11), and HPV-16 codon-optimized L1 ("l1optmzd"; SEQ ID No. 1). The sequences were aligned using the Gene Runner™ program (Hastings Software) available through the website maintained by the National Center for Biotechnology Information (NCBI). Nucleotides which differ between the aligned sequences are boxed.

[0024] FIG. 2 shows a schematic flowchart of the steps in the weaning selection process of an insect cell line capable of growing in serum-free media as suspension cell cultures.

[0025] FIG. 3 shows a schematic flowchart of the steps in the protein secretion selection process of an insect cell line capable of growing in serum-free media as suspension cell cultures and of enhanced expression of extracellular recombinant proteins and virus-like particles.

[0026] FIG. 4 shows a photomicrograph of a confluent monolayer of Sf-9S insect cells grown in serum-free insect cell media (Sf-900 II SFM, GIBCO) visualized by inverted phase-contrast microscopy at 400x magnification using Kodachrome 100 color film (Kodak).

[0027] FIG. 5 shows a schematic flowchart of the basic steps in the production or manufacturing of purified HPV VLP products.

[0028] FIG. 6 shows a schematic flowchart of the steps in upstream processing of baculovirus infected insect cell suspensions for production of recombinant HPV L1 VLPs.

[0029] FIG. 7 shows a schematic flowchart of the steps in upstream processing of baculovirus-infected insect cell suspensions for production of recombinant HPV chimeric VLPs.

[0030] FIG. 8A shows a schematic flowchart of the downstream processing method for purification of recombinant HPV VLPs by continuous flow ultracentrifugation using linear sucrose gradients.

[0031] FIG. 8B shows a schematic flowchart of the downstream processing method for purification of recombinant HPV VLPs by column chromatography using ion exchange and affinity binding matrices.

[0032] FIG. 8C shows a schematic flowchart of the downstream processing method for purification of recombinant HPV VLPs by ultracentrifugation using discontinuous sucrose step gradients.

[0033] FIG. 9 shows a stained protein gel of the products of the invention (*i.e.*, baculovirus-derived recombinant HPV-16 L1 VLPs purified according to the methods of the present invention.

[0034] FIG. 10A shows proteins detected chromogenically on membranes by Western blot analysis of recombinant HPV-16 L1 VLPs purified according to the methods of the present invention and bound to polyclonal antisera to HPV-16 L1 protein (1:10,000).

[0035] FIG. 10B shows proteins detected chromogenically on membranes by Western blot analysis of recombinant HPV-16 L1 VLPs purified according to the methods of the present invention and bound to polyclonal antisera to Sf-9S insect cell proteins (1:500).

[0036] FIG. 10C shows proteins detected chromogenically on membranes by Western blot analysis of recombinant HPV-16 L1 VLPs purified according to the methods of the present invention and bound to polyclonal antisera to *Ac*MNPV wild-type baculovirus (1:500).

[0037] FIG. 11 shows a graph of the binding results of H16.V5 murine monoclonal antibody to conformational epitopes on untreated and Triton X-100-treated recombinant HPV-16 L1 VLPs purified and treated according to the methods of the present invention as measured by enzyme linked immunoadsorbent assay (ELISA) analysis.

[0038] FIG. 12 shows a chromatogram of a product of the invention, recombinant HPV-16 L1 VLPs purified according to the methods of the present invention, analyzed by analytical size exclusion chromatography.

[0039] FIG. 13 shows an electron micrograph of baculovirus-derived recombinant HPV-16 L1 VLPs purified according to the methods of the present invention, stained negatively with uranyl acetate, and magnified 36,000x. The bar scale is 50 nm.

V. DETAILED DESCRIPTION OF THE INVENTION

[0040] The invention, as disclosed and described herein, provides compositions and methods for detecting, preventing, ameliorating, or treating papillomavirus related diseases and disorders. The pharmaceutical composition of the invention contains recombinant viral proteins

that self assemble into virus-like particles (VLPs) exhibiting conformational antigenic epitopes capable of raising neutralizing antibodies. The VLPs of the invention are expressed from a host cell extracellulary, intracellularly, or both.

Definitions

[0041] The definitions used in this application are for illustrative purposes and do not limit the scope of the invention.

[0042] As used in herein, "virus-like particles" or "VLPs" refers to virus particles that self-assemble into intact virus structures comprised of capsid proteins such as papillomavirus L1 capsid proteins. VLPs are morphologically and antigenically similar to authentic virions, but do not contain genetic information sufficient to replicate and thus are non-infectious. VLPs are produced in suitable insect host cells (*i.e.*, yeast, mamalian, and insect host cells), wherein upon isolation and further purification under suitable conditions, are purified as intact VLPs.

[0043] As used herein, "chimeric VLP" refers to recombinant papillomavirus L1 capsid protein, or peptide fragment thereof, that encapsulates other papillomavirus gene products or heterologous gene products during self-assembly into virus-like particles. For example, gene products containing the HPV L2, E2, E6, and/or E7 and which become encapsulated into the HPV L1 VLPs are considered herein as chimeric VLPs.

[0044] As used herein, "L2 fusion protein" refers to a protein, or a peptide fragment thereof, encoded by a papillomavirus L2 scaffolding gene fused to papillomavirus or other viral genes including heterologous gene(s).

[0045] As used herein, "heterologous viral capsid genes" refers to viral genes encoding the major structural virion component from different viruses, for example, the rotavirus VP2, VP6, HPV-16 L2, and HPV-16 L1 genes.

[0046] As used herein, “protein” is used interchangeably with polypeptide, peptide and peptide fragments.

[0047] As used herein, “polynucleotide” includes cDNA, RNA, DNA/RNA hybrid, anti-sense RNA, ribozyme, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified to contain non-natural or derivatized, synthetic, or semi-synthetic nucleotide bases. Also, included within the scope of the invention are alterations of a wild type or synthetic gene, including but not limited to deletion, insertion, substitution of one or more nucleotides, or fusion to other polynucleotide sequences, provided that such changes in the primary sequence of the gene do not alter the expressed peptide ability to elicit protective immunity.

[0048] As used herein, “gene products” include any product that is produced in the course of the transcription, reverse-transcription, polymerization, translation, post-translation and/or expression of a nucleotide molecule. Gene products include, but are not limited to, proteins, polypeptides, peptides, or peptide fragments.

[0049] As used herein, "L1 protein" refers to the structural protein of papillomavirus L1 capsid genes and constitutes the major portion of the papillomavirus ("PV") capsid structure. This protein has reported application in the preparation of HPV vaccines and diagnostic reagents.

[0050] As used herein, "L2 protein" refers to the structural scaffolding protein of papillomavirus, which constitutes a minor portion of the papillomavirus capsid structure and facilitates the assembly of papillomavirus particles within cell nuclei.

[0051] As used herein,"L2/E7 protein" refers to a fusion protein, or a fragment thereof, encoded by a papillomavirus L2 scaffolding gene fused to a papillomavirus E7 transforming gene that may have one or more mutations.

[0052] As used herein, "L2/E7/E2 protein" refers to a fusion protein, or a fragment thereof, encoded by a papillomavirus L2 scaffolding gene fused to (a) papillomavirus E2 transactivation gene that may have mutations and (b) a papillomavirus E7 transforming gene. The fused gene includes one or more mutated genes.

[0053] As used herein, "L2/E6 protein" refers to a fusion protein, or a peptide fragment thereof, encoded by a papillomavirus L2 scaffolding gene fused to a papillomavirus E6 transforming gene that may have one or more mutations.

[0054] As disclosed herein, “mutation” includes substitutions, transversions, transitions, transpositions, reversions, deletions, insertions, or other events that may have improved desired activity, or a decreased undesirable activity of the gene. Mutation encompasses null mutations in natural virus isolates or in synthesized genes that may change the primary amino acid sequences of the expressed protein but do not affect the self-assembly of capsid proteins, and antigenicity or immunogenicity of VLPs or chimeric VLPs.

[0055] As disclosed herein, “substantially homologous sequences” include those sequences which have at least about 50%, homology, preferably at least about 60-70 %, more

preferably at least about 70-80% homology, and most preferably at least about 95% or more homology to the codon optimized polynucleotides of the invention.

[0056] As used herein "vaccine" refers to compositions that result in both active and passive immunizations. Both polynucleotides and their expressed gene products are used as vaccines.

[0057] As used herein "biologically active fragments" refer to fragments exhibiting activity similar, but not necessarily identical, to an activity of the viral polypeptide of the present invention. The biologically active fragments may have improved desirable activity, or a decreased undesirable activity.

[0058] As used herein "polypeptides" include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptide, homodimers, heterodimers, variants of the polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, agonists, antagonists, or antibody of the polypeptide, among others. The polypeptides of the invention are natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

[0059] The terms "amino acid" or "amino acid sequence," as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to

naturally occurring or synthetic molecules. In this context, "fragments," "immunogenic fragments," or "antigenic fragments" refer to fragments of viral proteins which are preferably at least 5 to about 15 amino acids or more in length, and which retain some biological activity or immunological activity of the viral protein.

[0060] As used herein, "purity" refers to the amount of intact VLPs present in a final product of the invention.

[0061] As used herein, "yield" refers to the amount of purified intact VLPs as a function of the wet weight or the number of initial cells infected with recombinant baculoviruses expressing VLPs. For example, a preferred yield of intact VLPs is greater than about 10 mg of VLPs per 10^9 host cells.

[0062] As used herein, “antigenic characteristic(s)” refers to the ability of HPV VLPs to bind or cross-react with antisera generated against wild-type HPV virions of the same genotype. Antisera generated by immunization of animals or humans with HPV VLPs produced according to the present invention contains immunoglobulin molecules that share binding sites of native HPV virions with antisera from humans infected with HPV of the same genotype.

1. Sf-9S Cell Line

[0063] According to one aspect of the invention described herein, there is provided a novel cell line designated as Sf-9S, which was deposited as cell line ATCC PTA-4047 on February 4, 2002, under the Budapest Treaty, with the American Type Culture Collection (ATCC), located at 10801 University Boulevard, Manassas, Virginia 20110. The Sf-9S cell line

is derived from the parent *S. frugiperda* Sf-9 cell line (ATCC CRL-1771) and established by clonal selection based on serum-independent growth. This cell line is used as a host cell substrate in a single cell suspension maintained at a large manufacturing scale. The Sf-9S cell line of the invention is capable of enhanced expression of recombinant gene products. The designations "Sf-9S" and "ATCC PTA-4047" are used herein interchangeably, and refer to the same cell line.

[0064] According to one embodiment of the invention, there is provided a process for developing cell lines from the parent cell line Sf-9S. The first step of this process involves progressive weaning of cells from serum-containing media to serum-free media. Master and working cell banks of the cell line are constructed and qualified according to safety, identity, and biological criteria or specifications. Prior to commencement of the clonal selection process, master and working cell banks of the parental cell line Sf-9 cells are cultivated as monolayer cultures for at least 10, preferably at least 20, and more preferably at least 30 passages in Grace's insect media (Life Technologies, Grand Island, N.Y. 14072) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, N.Y. 14072). A master cell bank of Sf-9 cells is stored in conditioned serum-containing media at –70°C and in liquid nitrogen. A working cell bank is established from a single cryovial of the Sf-9 master cell bank and cultivated in serum-containing insect media for multiple cell passages. The clonal selection process, according to the invention, includes several rounds as demonstrated in FIG 2.

[0065] The method of clonal selection according to the invention described herein includes generally weaning a plurality of cells from serum-dependence to obtain at least one cell that can grow in serum-free medium.

[0066] According to another aspect, the invention provides a process for producing a cell line comprising one or more of the following steps: (a) plating a plurality of cells in wells containing serum-containing medium, one cell per well; (b) culturing the cell in each separate well; (c) identifying each well with replicating cells; (d) culturing the replicating cells into replica-plating wells; (e) changing the medium in each identified well with replicating cells by increasing the proportion of serum-free medium to serum-containing medium; (f) repeating identifying, culturing, and medium-changing of steps (c) – (e) until the medium for each well is approximately 100% serum-free; (g) harvesting the cells from each serum-free well; and (h) culturing the harvested cells in suspension. Suspension cultures of harvested cells that grow to a predetermined cell density for multiple passages are designated serum-free cell clones.

[0067] According to another embodiment, the method of clonal selection of the invention includes at least one of the following steps. First, cell clones capable of growing in commercial serum-free media as suspension cultures are isolated from monolayer cultures of parent Sf-9 cells dependent on serum-containing media by sequential weaning of parent cells from serum-containing media. According to one embodiment depicted in FIG. 2, Sf-9 parent cells are prepared at step **201** for sequential serum-weaning. In this embodiment, monolayer and suspension cultures of Sf-9 cells are grown at about 26–28°C in a dry environment. Shaker suspension cultures are agitated at about 100–150 rpm in a standard orbital or platform shaker incubator and stir flask suspension cultures are stirred at about 25–75 rpm on a standard laboratory magnetic stirrer.

[0068] In step **202**, cell aliquots are dispensed from a cell suspension (one cell per aliquot) of the parental cell line in serum-containing media into wells of 96-well dishes at a ratio

of one aliquot per well. Following cell attachment, cell acclimation to wells, and exclusion of wells with no cells and wells with more than one cell, in step **202** the media is changed from serum-containing media (100%) to a media mixture comprised of 75% serum-containing media and 25% serum-free media. In step **203**, cells are cultured in "75/25" media mixture for approximately one to two weeks. Wells that initially contain only one cell per well and demonstrate cell growth and replication (*i.e.*, four to five cells) after step **203** are subjected in step **204** to another media change to a mixture comprising 50% serum-containing media and 50% serum-free media. In step **205**, cells in the "50/50" media mixture of step **204** are allowed to grow for approximately another one to two weeks. The media mixture is changed again in step **206**, to a mixture comprising 25% serum-containing media and 75% serum-free media. In step **207**, cells are allowed to grow and replicate in the new "25/75" media mixture of step **206**. After another two to four weeks, in step **208** the media is changed in wells containing growing cells to a final media comprising serum-free media (100%).

[0069] During each step of the weaning process depicted in FIG. 2, a majority of the cells, for example about 95% of the cells or more, do not survive the reduction in serum. While not wanting to be bound by this theory, it is believed that this high level of cell death creates a selective pressure to permit development of a new cell phenotype. In step **209**, cells from wells that demonstrate continuous cell growth and replication are harvested by vigorous aspiration with serum-free media. In step **210**, the harvested cells are seeded into larger culture flasks (*i.e.*, 75 or 150 cm^2 T-flasks), and, in step **211**, the suspension cultures are grown. When greater than 4×10^6 cells with a viability > 95% is obtained, cells are harvested in step **211** and seeded into shaker or stir flasks as suspension cultures with a starting cell density, for example, about 0.2 -

0.5 x 10^6 cells/ml and a minimal ratio, for example, about 2.5 for total vessel capacity to total volume of culture media. In step **212**, cell clones that grow exponentially to a saturation cell density of greater than 6 x 10^6 cells/ml in serum-free media are selected, expanded, and frozen.

[0070] Finally, one cell clone is selected, passaged for at least 10, preferably at least 20, and more preferably more than 30 times as a suspension culture in serum-free media at a split ratio of at least 1:10, and established as a cell line. This serum-independent cell line is used to establish a master cell bank and subsequent working cell banks.

1.1 Sf-9S Cells

[0071] According to another aspect of the invention described herein, there are provided host cells that express one or more recombinant gene products with an enhanced yield. Insect host cells include, for example, Lepidopteran insect cells, and particularly preferred are *Spodoptera frugiperda*, *Bombyx mori*, *Heliothis virescens*, *Heliothis zea*, *Mamestra brassicas*, *Estigmene acrea* or Trichoplusia insect cells. Non-limiting examples of insect cell lines include, for example, Sf21, Sf9, High Five (BT1-TN-5B1-4), BT1-Ea88, Tn-368, mb0507, Tn mg-1, and Tn Ap2, among others.

[0072] In addition to the serum-weaning process described above, the Sf-9S cells of the present invention have undergone a recombinant peptide secretion selection process. An example of the process of the recombinant peptide secretion selection, according to the invention, is demonstrated in FIG. 3. The Sf-9S cells express extracellularly a foreign recombinant protein with an enhanced yield.

[0073] According to one embodiment of the invention, the cells are infected with a recombinant Baculovirus vector to express recombinant proteins or polypeptides of medical, pharmaceutical, or veterinary importance. Baculoviruses including *Autographa californica* multinucleocapsid nuclear polyhedrovirus (*Ac*MNPV) are propagated in cell lines derived from larval tissues of insects of the Lepidopteran insect family. General methods for handling and preparing baculovirus vectors and baculovirus DNA, as well as insect cell culture procedures, are outlined for example in O'Reilly *et al.*, 1994; Vaughn, J. 1999, *supra*; Frieson *et al.* 1986. In: The *Molecular Biology of Baculoviruses*, Doerfler *et al.*, Eds. Springer-Verlag, Berlin, pages 31-49; Kool *et al*, 1993. *Arch. Virol.* 130: 1-16, incorporated herein by reference in their entirety.

[0074] In one embodiment, polynucleotide molecules, including chimeric and heterologous polynucleotides, which encode a foreign peptide of interest, are inserted into the baculovirus genome operably coupled to or under the control of the polyhedrin or other Baculovirus promoters. The recombinant baculovirus vector is then used to infect a host cell. The foreign peptide or protein is expressed upon culture of the cells infected with the recombinant virus.

[0075] In another embodiment, the invention provides a method for producing a selected foreign protein in an insect cell. The method comprises preparing infected insect cells that express at least a first recombinant viral protein, and infecting the cell with a baculovirus comprising an expression vector that encodes a second recombinant viral protein. The first, or the second viral proteins, or both are, for example, viral capsid proteins including heterologous peptides and chimeric peptides. The cells produced according to the method disclosed herein

produce substantially high yields of recombinant baculoviruses expressing the desired recombinant peptides.

[0076] The insect cells of the invention have passed through a recombinant peptide secretion selection. As described herein, the process of recombinant peptide secretion selection includes one or more of the following steps. Cells from a serum-weaned clone are infected with a first baculovirus expressing a first recombinant protein. Cells capable of secreting high levels of the first recombinant protein are selected further for infection with a second baculovirus expressing a second recombinant protein. Cells from a clone that secretes high levels of both recombinant proteins independently are passaged further to establish the Sf-9S cell line of the present invention.

[0077] According to a preferred embodiment, the first recombinant protein or the second recombinant protein, or both, is a viral capsid protein that self-assembles into virus-like particles. In a more preferred embodiment, the virus-like particles are derived from viral capsid proteins of an enveloped virus, or a non-enveloped virus, including, but not limited to, an influenza virus, a hepatitis C virus, a retrovirus such as a human immunodeficiency virus, a calicivirus, a hepatitis E virus, a papillomavirus, or a combination thereof. In a most preferred embodiment of the invention, the virus-like particles are derived from human papillomavirus.

[0078] According to a preferred embodiment of the invention, the Sf-9S cells support intracellular, and preferably extracellular, expression of recombinant proteins and macromolecules. More preferably, infected Sf-9S cells extracellularly express viral capsid proteins that self assemble into VLPs. Virus-like particles typically self assemble in the cell and remain intracellular; therefore isolation of these particles requires processes of cell disruption

and protein solublilization with the accompanying risks of VLP disruption, proteolysis and contamination of the end product. Accordingly, the infected cells of the invention that afford self-assembly of viral capsid antigens into VLPs and facilitate secretion of VLPs extracellularly are highly desirable.

[0079] An example of a process for recombinant peptide secretion selection as depicted in FIG. 3 is described below. FIG. 3 demonstrates that additional rounds of clonal selection are used to obtain cells capable of enhanced secretion of recombinant proteins. In step **301,** cell aliquots from a cell suspension (one cell per aliquot) of the parent serum-free cell clone (*i.e.*, a cell line from one of the serum-free cell clones selected in step **213** of FIG. 2) are replica-plated into each well of 96-well plates at a ratio of one cell per well. In step **302,** wells containing a single cell from the original seeding are identified and grown to confluency. Upon confluency, cells from wells identified as single cell wells are subcultured into replica plates in step **303.** Cells in replica plates are grown at step **304** to confluency and infected with a first recombinant baculovirus expressing first virus capsid proteins that are capable of self-assembly into virus-like particles.

[0080] During baculovirus infection, in step **305** the infected cells and extracellular media are harvested by centrifugation to isolate infected cells and extracellular media, heat-denatured under reduced conditions (> 75°C for 5 minutes in 1% sodium dodecyl sulfate (SDS) and 10 mM β-mercaptoethanol), and analyzed by SDS-PAGE and Western blot analyses with antisera to viral capsid proteins. In step **306,** cells in replica plates that contain cell clones exhibiting extracellular VLPs at levels higher than control Sf-9 cells are infected with a second baculovirus expressing the second viral capsid proteins that self-assemble into virus-like

particles. The infected cells and extracellular media from the second selection round are isolated in step **307** by centrifugation and analyzed by SDS-PAGE and Western blot analyses. The first and second viral capsid proteins are the same or different proteins and include, for example, rotavirus VP2, VP6, and HPV-16 L1, HPV-L2 proteins, among others.

[0081] The test results from the first and second rounds of selection (*i.e.* virus infections producing VLPs) are examined in step **308**. The cell clone exhibiting the highest levels of extracellular VLPs from both virus infections is chosen in step **310**. From the replica plate, cells of the selected cell clone exhibiting highest extracellular VLP levels are passaged repeatedly in suspension culture with serum-free insect cell media to establish a cell line. The cell line supports high levels of extracellular VLP production upon infection with recombinant baculoviruses expressing viral capsid proteins that self-assemble into virus-like particles. Thus, in one embodiment, the clone selected in step **310** is processed again according to steps **304-309** with recombinant baculovirus expressing HPV-16 L1 capsid proteins. The cell clone that produces the highest levels of extracellular VLPs for both sets of viral capsid proteins is chosen in step **311** to establish a cell line capable of producing extracellular VLPs.

[0082] Master cell banks of Sf-9S cells are established, for example, from a single cell passage of the new cell line grown in suspension culture of serum-free medium and stored at -70°C in liquid nitrogen in a cryopreservation freezing media containing fresh serum-free media, conditioned serum-free media, and dimethyl sulfoxide. Working cell banks are developed, for example, from single cryovials of the master cell bank, subjected to safety and biological testing for qualification as a host cell substrate for manufacturing of recombinant protein products, and

stored at –70°C in liquid nitrogen in cryovials in cryopreservation freezing media as described above.

[0083] The Sf-9S cell line of the present invention demonstrate one or more of the following properties: (1) they replicate in serum-free media; (2) they are genetically distinct from parent Sf-9 parent cell line; (3) they grow as single cells in suspension cultures; (4) they demonstrate cell division rate of approximately 18-24 hours; (5) they demonstrate high cell viability (more than 95%) upon continuous cell culture for more than one year; (6) they constitute a cell substrate for *Autographa californica* baculoviruses to produce high-titered virus stocks (more than 10^7 plaque forming units (pfu)/ml); (7) they are suitable for recombinant protein expression and production from baculovirus vectors; (8) they are suitable host cell substrates for agarose plaque assays to titer baculovirus stocks; (9) they are compliant with recognized identity and safety guidelines; (10) they are suitable cell substrates for large-scale manufacturing of human and animal biological products including vaccines, therapeutics, and diagnostic reagents; (11) they are suitable cell substrates for transfection of genes in recombinant baculovirus transfer vectors and/or bacmids to produce recombinant baculoviruses, and (12) they produce high levels of extracellular VLPs from baculoviruses expressing viral capsid proteins that self-assemble into VLPs of non-enveloped viruses such as rotaviruses, caliciviruses, hepatitis E virus, and human papillomaviruses and of enveloped viruses such as influenza virus, hepatitis C virus, and human immunodeficiency virus.

[0084] In FIG. 4, a confluent monolayer of Sf-9S cells grown in serum-free insect cell media is shown at 400x magnification using a phase-contrast microscope. The cuboidal and

fibroblastic cell morphologies of the cell line are displayed. The cell morphology of Sf-9S cells changes from fibroblastic to cuboidal, as the monolayer becomes confluent.

[0085] Safety testing of the Sf-9S cell line produced according to the present invention and deposited at the ATCC may be performed in accordance with United States federal regulatory guidelines and include microbial sterility, mycoplasma and spiroplasma growth, endotoxins, adventitious agents (in vitro and in vivo assays), and electron microscopic examination for type C endogenous retrovirus particles. The cell identity of the Sf-9S cell line was shown by karyology and isotype enzyme analyses, to be *S. frugiperda* insect species with the typical polyploid chromosomal pattern distinct from mammalian cells.

1. Expression Systems

[0086] The expression vector of the invention is a baculovirus vector. For baculovirus vectors and baculovirus DNA, as well as insect cell culture procedures, *see*, for example in O'Reilly *et al.* 1994, incorporated herein by reference in its entirety. The baculovirus vector construct of the invention preferably contains additional elements, such as an origin of replication, one or more selectable markers allowing amplification in the alternative hosts, such as yeast cells and insect cells.

[0087] Host cells are infected, transfected, or genetically transformed to incorporate codon-optimized polynucleotides and express polypeptides of the present invention. The recombinant vectors containing a polynucleotide of interest are introduced into the host cell by any of a number of appropriate means, including infection (where the vector is an infectious agent, such as a viral or baculovirus genome), transduction, transfection, transformation,

electroporation, microprojectile bombardment, lipofection; or a combination thereof. A preferred method of genetic transformation of the host cells, according to the invention described herein, is infection.

[0088] In certain embodiments, there are provided baculovirus vectors that contain cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors are either supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host. Host cells are infected with baculovirus vectors comprising codon optimized polynucleotides to express polypeptides.

[0089] The polynucleotides are introduced alone or with other polynucleotides. Such other polynucleotides are introduced independently, co-introduced or introduced joined to the polynucleotides of the invention. Thus, for instance, a polynucleotides (*i.e.*, L1 gene) is transfected into host cells with another, separate polynucleotide (*i.e.*, L2 or fusion L2 genes) using standard techniques for co-transfection and selection. In another embodiment, the polynucleotides encoding L1 capsid protein and the polynucleotides encoding L2 protein or an L2 fusion protein are present on two mutually compatible baculovirus expression vectors which are each under the control of their own promoter.

3. Codon-Optimized Polynucleotides Encoding HPV Polypeptides

[0090] This invention also encompasses nucleic acid sequences that correspond to, and code for the HPV polypeptides. Nucleic acid sequences are synthesized using automated systems well known in the art. Either the entire sequence is synthesized or a series of smaller

oligonucleotides are made and subsequently ligated together to yield the full-length sequence. Alternatively, the nucleic acid sequence is derived from a gene bank using oligonucleotides probes designed based on the N-terminal amino acid sequence and well known techniques for cloning genetic material.

[0091] In addition, the codon-optimized polynucleotides comprising unusual bases, such as inosine, or modified bases, such as tritylated bases of 8-amino adenine bases, to name just a few are polynucleotides, the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "codon-optimized polynucleotide", as it is employed herein, embraces such chemically, enzymatically or metabolically modified forms of polynucleotide.

[0092] The codon-optimized polynucleotides of the present invention encode, for example, the coding sequence for the mature polypeptide, the coding sequence for the mature polypeptide and additional coding sequences, and the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences. Examples of additional coding sequences include, but are not limited to, sequences encoding a leader or secretory sequence, such as a pre-, pro-, or prepro-protein sequences. Examples of additional non-coding sequences include, but are not limited to, introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription and mRNA processing, including splicing and polyadenylation signals, for example, for ribosome binding and stability of mRNA.

[0093] The codon-modified polynucleotides also encode a polypeptide which is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to

the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, may facilitate protein trafficking, may prolong or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. The additional amino acids may be processed away from the mature protein by cellular enzymes.

[0094] In sum, a codon-optimized polynucleotide of the present invention encodes, for example, a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences which are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

[0095] According to one embodiment of the invention, there are provided codon-optimized polynucleotides that encode one or more foreign proteins. In this embodiment, the codon optimization of the invention is based on the following criteria: (1) abundance of aminoacyl-tRNAs for a particular codon in Lepidopteran species of insect cells for a given amino acid as described by Levin and Whittome (2000), (2) maintenance of GC-AT ratio in L1 gene sequence is approximately 1:1, (3) minimal introduction of palindromic or stem-loop DNA structures, and (4) minimal introduction of transcription and post-transcription repressor element sequences.

[0096] The optimized genes sequence is synthesized *in vitro,* for example, as overlapping oligonucleotides, cloned, and expressed in a host cell. Cloning and expression of the codon

modified viral genes were achieved following the methods known in the art and exemplified at Examples 3 and 4 herein.

[0097] In a preferred embodiment of the invention, polynucleotides encoding a viral gene, for example HPV genes, are optimized for expression in a baculovirus-infected insect cell, comprising one or more of the following steps (a) replacing nucleotide sequences of codons in the gene that are underutilized in insect cells of Lepidopteran species with sequences of preferred codons in insect cells; and (b) for each amino acid encoded by this modified nucleotide sequence, if a plurality of codons for the same amino acid is preferred in insect cells, then the nucleotide sequence of the modified gene is changed further by selecting a codon from preferred codons for a amino acid so that (i) the ratio of GC nucleotides to AT nucleotides in the sequence trends toward 1:1; (ii) the number of palindromic and stem-loop structures is minimized unless indicated otherwise for functional activity; and (iii) the number of transcription and/or post-transcription repressor elements in the sequence is minimized.

[0098] This method was used to develop the codon-optimized polynucleotides encoding HPV L1 (used to generate L1 VLPs), and HPV L2 (including wildtype L2), L2/E7, L2/E7/E2, and L2/E6 (used to generate chimeric VLPs). The nucleic acid sequences of the codon optimized HPV L1, HPV L2, and HPV L2 fusion genes are represented herein as HPV L1 (SEQ ID NO. 1), HPV L2 (SEQ ID NO. 2,), HPV L2/E7 (SEQ ID NO. 3), HPV L2/E7/E2 (SEQ ID NO. 4), and HPV L2/E6 (SEQ ID NO. 5), respectively.

[0099] The method of codon optimization of the invention, as described herein, is used to, *inter alia*, optimize the expression of variety of enveloped and non-enveloped viral genes expressed in insect cells.

[00100] The codon-optimized polynucleotides of the invention include "variant(s)" of polynucleotides, or polypeptides as the term is used herein. Variants include polynucleotides that differ in nucleotide sequence from another reference polynucleotide. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. As noted below, changes in the nucleotide sequence of the variant amy be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type, a variant will encode a polypeptide with the same amino acid sequence as the reference.

[00101] Changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. According to a preferred embodiment of the invention, there are no alterations in the amino acid sequence of the polypeptide encoded by the codon optimized polyneucleotide of the invention, as compared with the amino acid sequence of the wild type peptide.

[00102] The present invention further relates to polynucleotides that hybridize to the herein-described sequences. The term "hybridization under stringent conditions" according to the present invention is used as described by Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press 1.101-1.104, 1989. Preferably, a stringent hybridization according to the present invention is given when after washing for an hour with 1% SSC and 0.1 % SDC at 50°C, preferably at 55° C, more preferably at 62° C, most preferably at 68°C a positive hybridization signal is still observed. A polynucleotide sequence which

hybridizes under such washing conditions with the nucleotide sequence shown in any sequence disclosed herein or with a nucleotide sequence corresponding thereto within the degeneration of the genetic code is a nucleotide sequence according to the invention.

[00103] The codon-optimized polynucleotides of the invention include polynucleotide sequences that have at least about 50%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more nucleotide sequence identity to the codon optimized polynucleotides or a transcriptionally active fragment thereof. To determine the percent identity of two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*i.e.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second nucleic acid sequence). The amino acid residue or nucleotides at corresponding amino acid or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical overlapping positions/total # of positions x 100). In one embodiment, the two sequences are the same length.

[00104] The determination of percent identity between two sequences also can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST program of Altschul, *et al.*, 1990, *J. Mol. Biol.* 215:403-410.

BLAST nucleotide searches can be performed with the NBLAST program program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. The BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, *Nucleic Acids Res.*25:3389-3402.

[00105] Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST and PSI-Blast programs, the default parameters of the respective programs (*i.e.*, XBLAST and NBLAST program can be used (*see*, HTTP://WWW.NCBI.NLM.NIH.GOV). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences of a PAM 120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used. In an alternate embodiment, alignments can be obtained using the NA_MULTIPLE_ALIGNMENT 1.0 program, using a GapWeight of 5 and a GapLengthWeight of 1.

4. Recombinant HPV Polypeptides

[00106] In general, as used herein, the term polypeptide encompasses variety of modifications, particularly those that are present in polypeptides expressed by polynucleotides in a host cell. It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many

amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques.

[00107] It will be appreciated, that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural processes and by entirely synthetic methods, as well.

[00108] Modifications occur anywhere in a polypeptide, including the peptide backbone, the amino acid side chains and the amino or carboxyl termini. Blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, occur in a natural or synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. In general, the nature and extent of the modifications are determined by the host cell's post-translational modification capacity and the modification signals present in the polypeptide amino acid sequence. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a polypeptide.

[00109] The recombinant foreign polypeptide according to the invention includes truncated and/or N-terminally or C-terminally extended forms of the polypeptide, analogs having amino acid substitutions, additions and/or deletions, allelic variants and derivatives of the polypeptide, so long as their sequences are substantially homologous to the native antigenic viral polypeptide.

[00110] Specifically, as will be appreciated by those skilled in the art, the recombinant viral polypeptides of the invention include those polypeptides having slight variations in amino acid sequences or other properties. Such variations may arise naturally as allelic variations, as disclosed above, due to genetic polymorphism, for example, or may be produced by human intervention (*i.e.*, by mutagenesis of cloned DNA sequences), such as induced point, deletion, insertion and substitution mutants. Minor changes in amino acid sequence are generally preferred, such as conservative amino acid replacements, small internal deletions or insertions, and additions or deletions at the ends of the molecules.

[00111] Substitutions may be designed based on, for example, the model of Dayhoff, *et al.*, *Atlas of Protein Sequence and Structure*, Nat'l Biomed. Res. Found. Washington, D.C., 1978. These modifications can result in changes in the amino acid sequence, provide silent mutations, modify a restriction site, or provide other specific mutations. The recombinant viral polypeptides may comprise one or more selected antigenic determinants of the viral polypeptide peptides, possess catalytic activity exhibited by their native protein or alternatively lack such activity.

The conserved and variable sequence regions of a viral polypeptide and the homology thereof can be determined by techniques known to the skilled artisan, such as sequence alignment techniques. For example, the determination of percent identity between two sequences can also be accomplished using a mathematical algorithm, as described above.

4.1 Virus-Like Particles (VLPs)

[00112] Virus-like particles (VLPs) are the expressed product of the codon-optimized polynucleotides of the invention. The capsid protein encoded by the codon-optimized polynucleotide of the invention is capable of self assembly into virus-like particles that exhibit conformational antigenic epitopes capable of eliciting neutralizing antibodies in a subject.

[00113] Encompassed within the scope of the invention are VLPs comprising capsid protein of non-enveloped and enveloped viruses, including rotaviruses, caliciviruses, hepatitis E virus, and human papillomaviruses, influenza virus, hepatitis C virus, and retrovirus, including human immunodeficiency virus. Preferably, the VLPs comprise Papillomavirus L1 capsid protein.

[00114] Also encompassed within the scope of the invention are VLPs derived from different species and genotypes of papillomaviruses. Papillomaviruses of the invention are, for example, from human, simian, bovine, or other origins. Preferably, the papillomavirus of the invention is a human papillomavirus (HPV). More than 100 different human papillomavirus (HPV) genotypes have been isolated. Human papillomavirus genotypes include, but are not limited to, HPV-16, HPV-18, and HPV-45 for high-risk cervical cancers, HPV-31, HPV-33, HPV-35, HPV- 51, and HPV-52 for intermediate-risk cervical cancers, and HPV-6, HPV-11, HPV-42, HPV-43, and HPV-44 for low-risk cervical cancer and anogenital lesions (Bosch *et al.*, 1995; Walboomers *et al.*, 1999). HPV genotypes are also disclosed in PCT publication No. WO 92/16636 (Boursnell *et al.*, 1992), incorporated herein by reference in its entirety. HPV-16 is a preferred genotype of the invention.

4.2. Chimeric VLPs

[00115] Chimeric VLPs refer to viral capsid proteins that encapsulate other viral proteins or heterologous gene products. A preferred chimeric VLP according to the invention is a papillomavirus L1 capsid protein, or peptide fragment thereof, which encapsulate other papillomavirus gene products or heterologous gene products during self-assembly into virus-like particles. For example, gene products containing the HPV L2, E2, E6, and/or E7 gene products become encapsulated into the HPV L1 VLPs and are considered herein as chimeric VLPs.

4.2.1. Fusion Proteins

[00116] As one of skill in the art will appreciate, and as discussed above, the HPV polypeptide of the invention can be fused to heterologous polypeptide sequences. For example, the HPV L2 polypeptide of the present invention (including fragments or variants thereof) may be fused to one or more additional HPV polypeptide or other non-enveloped virus or enveloped virus polypeptides.

[00117] Additional fusion proteins of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. *See*, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten *et al., Curr. Opinion Biotechnol.* 8:724-33, 1997; Harayama, *Trends Biotechnol.* 16(2):76-82, 1998; Hansson, *et al., J. Mol. Biol.* 287:265-76, 1999; and Lorenzo and Blasco, *Biotechniques* 24(2):308-13,

1998 (each of these patents and publications are hereby incorporated by reference in its entirety). DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence.

In another embodiment, polynucleotides of the invention, or the encoded polypeptides, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of a polynucleotide encoding a polypeptide of the invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

[00118] Nucleic acids encoding the above fusion polypeptides can be recombined with a gene of interest as an epitope tag (*i.e.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. (*See*, for example, Janknecht *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8972-897, 1991). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix-binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni2+ nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

[00119] The cloning and expression of the L2 and L2 fusion genes can be achieved following the methods known in the art. One example of such methods is exemplified at Example 4 herein.

5. *Production, Isolation and Purification of Recombinant VLPs*

[00120] The present invention, as disclosed and described herein, provides methods for production, isolation and purification of recombinant viral gene products that are capable of self-assembly into intact virus-like particles exhibiting conformational antigenic epitopes. The virus-like particles of the invention can be prepared as pharmaceutical compositions or vaccines to induce a high-titer neutralizing antibody response in vertebrate animals. The self-assembling capsid proteins can also be used as elements of diagnostic immunoassay procedures for papillomavirus infection.

5.1. *Production of VLPs*

[00121] The present invention encompasses process for producing a recombinant virus-like particle including, for example, a virus-like particle of an enveloped virus or a non-enveloped virus, by infecting a permissive insect cell with a recombinant baculovirus that encodes viral capsid and/or envelope genes of one or more viruses. In one embodiment, the invention provides methods for harvesting and purifying HPV VLPs, including HPV chimeric VLPs from infected insect cells or other host cells. The baculovirus-infected cell expresses viral capsid and/or envelope proteins that self-assemble into virus-like particles. The VLPs are expressed intracellularly, extracellularly, or both.

[00122] In a preferred embodiment of the invention, the VLPs are produced extracellularly.

[00123] According to another embodiment of the invention, there is provided a method for the production of intracellular and extracellular HPV VLPs. In this embodiment as depicted step **505** of FIG. 5, production of intracellular and extracellular HPV-16 L1 VLPs begins with high multiplicity infection of log phase Sf-9S cells with an aliquot of a working virus stock, as depicted in step **504** of FIG. 5, of baculoviruses expressing HPV L1 capsid proteins. The virus infection can be monitored daily by the trypan blue exclusion method for cytopathic effects, cell viability, and cell density and by SDS-PAGE and Western blot analyses of recombinant HPV L1 capsid proteins in infected cells and extracellular media. At peak recombinant HPV L1 gene expression, infected cells and extracellular media containing intracellular and extracellular HPV L1 VLPs, respectively, are harvested and processed to obtain purified HPV L1 VLP products as outlined in step **506** of FIG. 5 and in depicted in more detail in FIG. 6 described below.

5.2. Production of Chimeric VLPs

[00124] According to yet another embodiment of the invention, there is provided a method for production of chimeric VLPs. In a preferred embodiment, the chimeric VLPs are HPV chimeric VLPs. In one embodiment, as depicted in FIG. 5, production of intracellular HPV chimeric VLPs begins with high multiplicity infection of log phase Sf-9S insect cells in suspension cultures containing serum-free insect cell medium with aliquots of HPV L1 and L2 fusion working virus stocks (*i.e.*, recombinant baculoviruses expressing L1 capsid protein and L2 fusion protein, respectively).

[00125] The ratio of co-infecting viruses is approximately at least about 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, or more. In a preferred embodiment the ratio of co-infecting viruses is approximately at least about 1:3, 1:5 or 1:10 (L1 to L2 virus). The virus infection is monitored daily by the trypan blue exclusion method for cytopathic effects, cell viability, and cell density and by SDS-PAGE and Western blot analyses of recombinant HPV L1 and L2 fusion proteins in infected cells. At peak recombinant HPV L1 and L2 fusion gene expression, infected cells containing intracellular HPV chimeric VLPs are harvested and processed to obtain purified HPV chimeric VLPs products as outlined in FIG. 5 and described in more detail below with reference to FIG. 7. This co-infection process may be used to manufacture recombinant papillomavirus chimeric VLPs of various viral genotypes including, but not limited to, those identified above associated with HPV infections and cancers.

5.3 *Upstream Processing of L1 VLPs*

[00126] The present invention also includes methods for upstream processing of transformed host cells including yeast, insect, and mammalian cells expressing recombinant VLPs. The recombinant VLPs are preferably L1 VLPs. More preferably the recombinant VLPs are HPV L1 VLPs.

[00127] In one embodiment, the process for purifying recombinant extracellular HPV L1 VLPs includes at least one of the steps of harvesting a cell suspension containing recombinant extracellular HPV L1 VLPs to produce a harvested supernatant, clarifying the harvested supernatant, concentrating the clarified supernatant, and dialyzing the concentrated supernatant.

[00128] In another embodiment, the process for purifying intracellular recombinant HPV L1 VLPs includes at least one of the steps of harvesting a cell suspension comprising infected cells containing intracellular recombinant papillomavirus L1 VLPs, and disrupting the harvested cells (which may have been resuspended in a buffer containing protease inhibitors) by, for example, sonication to produce crude infected cell lysates containing recombinant HPV L1 VLPs. The crude infected cell lysates is then clarified by, for example, centrifugation to produce a clarified supernatant containing recombinant HPV L1 VLPs. The clarified supernatant is then concentrated by, for example, ultrafiltration to produce a concentrate containing recombinant HPV L1 VLPs, and dialyzing concentrates, for example, against high salt buffers by ultrafiltration to produce a diafiltered crude product containing recombinant HPV L1 VLPs. The crude product is further processed by downstream processing as described below.

[00129] One example of the upstream processing of L1 VLPs is shown by Fig. 6. Recombinant HPV L1 VLPs from baculovirus-infected insect cells (step **601**) or from other cells expressing recombinant HPV VLPs are harvested by, for example, low-speed centrifugation (step **602**). In step **603** of the embodiment as depicted in FIG. 6, extracellular supernatants are separated from cell pellets by, for example, aspiration following centrifugation and, at step **604**, are clarified by, for example, centrifugation to remove large cell debris from extracellular recombinant HPV L1 VLPs. At step **605** of the embodiment depicted in FIG. 6, cell pellets containing intracellular recombinant HPV VLPs are discarded or resuspended in multifold cell volumes of buffer solution such as phosphate-buffered saline solution to produce a cell suspension. In step **606** of the embodiment depicted in FIG. 6, resuspended cells containing intracellular recombinant HPV L1 VLPs are disrupted with, for example, several pulses of

sonication to produce crude cell lysates without proteosome disruption. The cell sonicates containing HPV L1 VLPs are monitored for cell disruption by, for example, the trypan blue exclusion method. In step **607** of the embodiment depicted in FIG. 6, crude cell lysates containing intracellular HPV L1 VLPs are clarified by, for example, centrifugation to remove large cellular debris.

[00130] In another embodiment, clarified supernatants from supernatant media and cell lysates are combined as depicted in step **608** and concentrated multifold by, for example, ultrafiltration using hollow fiber filters (step **609**). The concentrates are dialyzed against multiple volumes of buffer by, for example, ultrafiltration using hollow fiber filters (step **610**).

[00131] Biological products including mock and/or wild type baculovirus-infected insect cells as described above may be used to prepare cell lysates containing host cells and/or baculovirus proteins. The proteins in the cell lysates are solubilized by, for example, sonication to disrupt cells and clarified by centrifugation as described above and mixed with Freund's or other adjuvant to produce immunogens.

[00132] Immunogenicity of the immunogens obtained above can be determined according to methods known in the art. For example, the immunogens can be administered at least once by intramuscular, subcutaneous, or intranasal routes into animals. After testing sera or immune cells from immunized animals for antigen specificity, the sera or immune cells from immunized animals were isolated following vaccination. The antibody titer specific for host cell and/or baculovirus proteins were determined by immunodetection methods such as ELISA or Western blot assays. The titered antisera can were usec in immunodetection assays such as Western blot analysis to determine the level of host cell and/or baculovirus present in recombinant protein

products derived from baculovirus-derived sources. T cell assays such as lumphocute proliferation assays and ELISPOT assays, whicha re used to determine the abundance of CD8+ cytotoxic T cells and the level of accompanying lymphokines and cytokines produced as a result of sensitization with the VLPs in this invention following immunization.

5.4 Upstream Processing of Chimeric VLPs

[00133] In another embodiment of the invention, methods for upstream processing of expressed VLPs are provided. In a preferred embodiment, the invention discloses the upstream processing of HPV VLPs and chimeric VLPs derived from insect cells co-infected with recombinant baculoviruses expressing L1 and L2 fusion genes.

[00134] In a specific embodiment, the upstream processing of chimeric VLPs generally includes one or more of the steps of harvesting a cell suspension comprising co-infected cells containing intracellular recombinant HPV chimeric VLPs, resuspending the harvested infected cells in a buffer containing a protease inhibitor, disrupting the resuspended infected cells by, for example, sonication, to produce crude infected cell lysates containing recombinant HPV chimeric VLPs, clarifying the crude infected cell lysates by, for example, centrifugation, to produce a clarified supernatant containing recombinant HPV chimeric VLPs, concentrating the clarified supernatants by, for example, ultrafiltration, to produce a concentrate containing recombinant HPV chimeric VLPs, and dialyzing concentrates against high salt buffers by, for example, ultrafiltration, to produce a diafiltered crude product containing recombinant HPV chimeric VLPs. The crude product thus obtained goes through downstream processing.

[00135] In a preferred embodiment, upstream processing of the invention is performed following steps of the method depicted in FIG. 7. Recombinant HPV chimeric VLPs are harvested from transformed cells, for example, baculovirus-infected insect cells, by, for example, low-speed centrifugation (step **702**). At step **703,** supernatant media are separated from cell pellets by, for example, aspiration following centrifugation. At step **704** supernatants media are discarded and cell pellets containing intracellular recombinant papillomavirus chimeric VLPs were resuspended in multiple cell volumes of a buffer solution containing protease-inhibitors that block the activity of at least one of the following protease classes: serine, aspartate, cysteine, and metallo. In step **705,** resuspended cells containing intracellular recombinant HPV chimeric VLPs are disrupted with, for example, several pulses of sonication to produce crude cell lysates without proteosome disruption that may cause proteolysis of chimeric L2 fusion proteins. The cell sonicates containing HPV chimeric VLPs are monitored for cell disruption by the trypan blue exclusion method. In step **706,** crude cell lysates containing intracellular HPV chimeric VLPs were clarified by, for example, centrifugation to remove large cellular debris. Clarified supernatants from cell lysates are concentrated multifold by ultrafiltration using hollow fiber filters (step **707**). The concentrates are dialyzed against multiple volumes of buffer containing protease inhibitors by ultrafiltration using hollow fiber filters (step **708**).

5.5 Downstream Processing of VLPs

[00136] In yet another embodiment of the invention, methods for downstream processing of expressed VLPs are provided. In a preferred embodiment, the invention discloses the downstream processing of HPV VLPs harvested from cells.

[00137] Downstream processing as depicted in FIGS. 5 and 8A - 8C includes one or more of the following steps: a linear sucrose gradient scheme (FIG. 8A), a chromatographic scheme (FIG. 8B), or a sucrose step gradient scheme (FIG. 8C). The product of these purification schemes yields recombinant HPV VLPs that are formulated to inactivate residual baculovirus contaminants by one or more of the following treatments: detergent treatment to remove process excipients; by ultrafiltration, to provide a buffer solution that promotes VLP stability; by diafiltration, to remove any microbial contaminants by terminal filtration.

5.5.1. Downstream Processing of VLPs: Linear Sucrose Gradient Scheme

[00138] The methods of the present invention encompasses downstream processing of crude materials containing recombinant VLPs by one or more of the following steps: sedimenting the recombinant VLPs through a first linear sucrose gradient (for example, by continuous flow ultracentrifugation), collecting fractions from the first gradient, identifying fractions containing recombinant VLPs, pooling fractions containing recombinant VLPs, rebanding pooled fractions through a second linear sucrose gradient, collecting fractions from a second gradient, and pooling fractions containing recombinant VLPs from the second gradient. In an embodiment, only one sedimentation step is used.

[00139] In one specific embodiment depicted in FIG. 8A, diafiltered concentrates (step **610** of FIG. 6 and step **708** of FIG. 7) or other materials containing VLPs are purified by continuous flow rate-zonal ultracentrifugation on linear sucrose gradients, based primarily on the mass and density of recombinant VLPs in sucrose (FIGS. 5 and 8A). For example, diafiltrates containing recombinant HPV VLPs are loaded under pressure onto approximately 0–65% linear

sucrose gradients in a vertical rotor accelerating at high speed in a continuous flow ultracentrifuge. The gradient is resolved by ultracentrifugation at high speed until recombinant HPV VLPs separate from baculovirus particles (step **802**). Gradient materials from the first round of sucrose gradients are monitored by ultraviolet light during collection in a fraction collector. Gradient fractions from the first round of linear sucrose gradients are analyzed by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins. Peak fractions containing HPV VLPs or their component proteins are pooled, diluted multifold with buffer solution, and are subjected to a second round of ultracentrifugation on linear sucrose gradients (step **803**).

[00140] In another embodiment, gradient materials from the second round of sucrose gradients are monitored by ultraviolet light during collection in a fraction collector. Gradient fractions from the second round of ultracentrifugation may be analyzed also by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins. Peak fractions containing HPV VLPs or their component proteins are pooled. The purified recombinant HPV VLPs in the pooled fractions are formulated as recombinant HPV VLP products.

5.5.2. Downstream Processing of VLPs: Chromatographic Scheme

[00141] The chromatographic method for downstream processing of VLPs, and preferably HPV VLPs includes at least one of the following three steps: adsorptive cation exchange chromatography using a pH gradient, affinity chromatography using heparin-like matrices for binding VLPs, and displacement anion exchange chromatography.

[00142] In the first chromatography step, diafiltered concentrates (step **610** of FIG. 6 and step **708** of FIG. 7) or other materials containing HPV papillomavirus VLPs, are loaded in the initial chromatographic step (step **805** of FIG.8B) onto a chromatography column containing a strong cation exchange chromatography resin with an exposed amino group such as Streamline SP (Amersham Biosciences) that is equilibrated with multiple volumes of loading buffer at low salt and a pH between 4.5 to 6.0. This step separates recombinant HPV VLPs from the bulk majority of host contaminant proteins and other molecules based on the isoelectric charge of HPV L1 proteins.

[00143] Following binding of diafiltrates or other materials containing recombinant papillomavirus VLPs to the charged resin, the bound column is washed with multiple volumes of loading buffer. In one embodiment, bound recombinant papillomavirus VLPs are eluted from the column resin using a low salt pH step gradient from 6.0 to 8.0. Elution fractions are analyzed by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins . Purification of chimeric VLPs by this chromatographic scheme has not been tried to date. In another embodiment, peak fractions containing VLPs or their component proteins are pooled.

[00144] In the second chromatography step (FIG. 8, step **806**), the pooled eluates from step **805** resulting from the cation exchange chromatography step or other materials containing recombinant papillomavirus VLPs are dialyzed against multiple volumes of affinity loading buffer by diafiltration. The dialysate is loaded onto a column containing heparin agarose or other molecules having an exposed carboxy group such as, for example, heparin sulfate glycans, glycoaminoglycans, $\alpha_6\beta_1$ integrin, $\alpha_6\beta_4$ integrin, syndecan 1, Matrex Cellufine Sulfate

(American Biosciences), or other heparin-like resins equilibrated with affinity loading buffer. Affinity chromatography using heparin serves as receptors for papillomavirus as binding matrices and affords high levels of specific and selective purification of recombinant HPV VLPs.

[00145] The bound column is washed with multiple volumes of affinity loading buffer. Bound proteins including recombinant papillomavirus VLPs, are eluted from the column resin using a linear salt gradient from approximately 300 mM to 2 M. Elution fractions are analyzed by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins. Peak fractions containing VLPs or their component proteins are pooled.

[00146] In the third chromatography step, pooled eluates from the affinity chromatography step (step **806**), that contain recombinant papillomavirus VLPs are dialyzed against multiple volumes of anion loading buffer by dialfiltration. The removal of small molecular weight molecules and residual host contaminant proteins in pooled affinity eluates or other material containing recombinant HPV VLPs is provided by anion exchange chromatography (FIG.8 step **807**) using a displacement polymer as a final polishing step based on the isoelectric point of HPV L1 proteins. The dialysate is loaded onto a column containing a strong anion exchange chromatography resin with an exposed carboxyl group such as, for example, Q Sepharose Fast Flow (FF) (Amersham Biosciences), Toyopearl Super Q-650 M (Tosoh Biosep), Q Sepharose FF, or Fractogel TMAE (USB) equilibrated with multiple volumes of anion loading buffer.

[00147] Bound proteins including recombinant HPV VLPs are displaced from the anion column resin with a linear gradient from approximately 0 to 5 mg/ml dextran sulfate (5000 MW). Elution fractions are analyzed by, for example, SDS-PAGE and Western blot analysis using

antisera against papillomavirus L1 capsid proteins. If required, peak fractions containing recombinant HPV VLPs or their component proteins are pooled and dialyzed by ultrafiltration against multiple volumes of high salt buffer to remove dextran sulfate.

5.5.3 Downstream Processing of VLPs: Sucrose Step Gradient Scheme

[00148] In yet another alternate embodiment of the present invention, diafiltered concentrates (step **610** of FIG. 6 and step **708** of FIG. 7) or other materials containing VLPs are purified by rate-zonal ultracentrifugation on discontinuous sucrose step gradients based primarily on mass and density of recombinant VLPs in sucrose.

[00149] The methods of the present invention encompasses downstream processing of recombinant VLPs and preferably recombinant HPV VLPs from crude materials by pelleting crude materials containing recombinant HPV VLPs through a sucrose cushion, resuspending the pelleted recombinant HPV VLPs, banding resuspended recombinant HPV VLPs by ultracentrifugation on discontinuous linear step gradients, collecting at least one bands containing recombinant HPV VLPs, and dialyzing banded material by diafiltration to remove sucrose.

[00150] In one embodiment depicted in FIG. 8C, diafiltrates containing recombinant HPV VLPs are loaded onto approximately 25% sucrose cushions in a swinging bucket rotor accelerating at high speed in an ultracentrifuge (step **809** of FIG. 8). The pellets at the bottom of the sucrose cushion are collected, while the sucrose cushion and load material are discarded. The sucrose cushion pellets are solubilized in buffer and loaded onto sucrose step gradients containing multiple steps comprising approximately 25 to 65% sucrose. The sucrose step

gradients are resolved by ultracentrifugation in a swinging bucket rotor at high speed until recombinant HPV VLPs are separated from baculovirus particles (step 810). Gradient materials from the first round of sucrose step gradients are monitored by ultraviolet light during collection in a fraction collector. Gradient fractions are analyzed by, for example, SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins.

[00151] Peak fractions containing HPV VLPs or their component proteins are pooled, diluted multifold with buffer solution, and optionally subjected to a second round of ultracentrifugation on sucrose step sucrose gradients (step 811). Gradient materials from the second round of sucrose step gradients are monitored by ultraviolet light during collection in a fraction collector. Gradient fractions from the second round of ultracentrifugation are analyzed by, for example, SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins. Peak fractions containing HPV VLPs or their component proteins are pooled. In an embodiment, the purified recombinant HPV VLPs in the pooled fractions are formulated as recombinant HPV VLP products.

5 6. Formulation of Papillomavirus VLP Products

[00152] According to the present invention as depicted in FIG. 5, pooled fractions or other material containing recombinant VLPs from the purification schemes described above may contain recombinant baculovirus particles, which are inactivated by treatment with, for example, a nonionic detergent, surfactants, ultraviolet light, or a combination thereof. In an embodiment of the present invention, a nonionic detergent, and a surfactant, such as Triton X-100, is added to the product containing recombinant HPV VLPs at a final concentration of approximately >0.1%.

The recombinant papillomavirus VLP mixture with detergent is incubated for at least approximately one hour to inactivate residual baculoviruses.

[00153] In an another embodiment, the recombinant HPV VLPs are irradiated with one or more rounds of ultraviolet (UV) light < 300 nm and then incubated in a nonionic detergent, or both. Multiple log reduction of the baculovirus is afforded by these treatments which may have additive or synergistic effect. The process for inactivating residual baculovirus products are also used for other recombinant protein products, including recombinant protein products comprising VLPs of virus types identified above. In addition, VLP products treated according to this process are dialyzed against a buffer in order to refold the conformational epitopes of the VLPs in the product.

[00154] In yet another embodiment, following the baculovirus inactivation treatment(s), recombinant HPV VLP products are dialyzed by ultrafiltration against multiple volumes of high salt buffer containing approximately >0.5 M sodium chloride at approximately neutral pH to remove process excipients such as sucrose, Triton X-100 detergent, and other molecules. Diafiltrates containing recombinant HPV VLP bulk products are filtered aseptically through a 0.2 μm membrane at ambient temperature to remove microbial contaminants. To maintain high levels of intact VLPs in the final bulk products, the filtered recombinant HPV VLPs are dispensed directly into sterilized 316 L stainless steel tanks, silanized borosilicate glass bottles, or polyethylene plastic bioprocess bags and stored at 2 – 8°C for < six (6) months, or at <-70°C for 2 years.

[00155] Bulk recombinant HPV VLP products made according to the present invention are formulated alone or with adjuvants such as, for example, Novasomes™ and micelle nanoparticles, among others. For monovalent products, bulk products containing one genotype of recombinant HPV VLPs are diluted with buffer solution to the appropriate antigen concentration such as 100 μg/ml, mixed with an adjuvant, adjusted for final pH and salt concentrations, filtered aseptically through 0.2 μm membranes, and dispensed into silanized borosilicate vials. For multivalent products, equal molar antigen concentrations of bulk products representing more than one genotype of recombinant HPV VLPs are formulated and processed into final container products as described above for monovalent products. Final container products are stored at 2 – 8°C for <6 months or <-70°C for extended time such as two years or less. Following qualification of final container products for purity, strength, identity, potency, and safety, final container products are used as pharmaceutical composition, prophylactic vaccines, or diagnostic reagents.

[00156] In one embodiment, prophylactic vaccines for the prevention of anal genital warts are formulated as mixtures of at least HPV-6 and/or HPV-11 L1 or chimeric VLPs. Prophylactic vaccines for the prevention of HPV-induced cervical cancer are formulated as mixtures of at least HPV-16, HPV-18, HPV-31, and/or HPV-33 L1 or chimeric VLPs. Therapeutics for treatment of anal genital warts are formulated as mixtures of at least HPV-6 and/or HPV–11 chimeric VLPs. Pharmaceutical compositions for treatment of HPV-induced cervical cancer are formulated as mixtures of at least HPV-16, HPV-18, HPV-31, and HPV-33 chimeric VLPs.

[00157] These and other products comprising recombinant VLPs made according to the present invention are administered by various parenteral and local routes including but not

limited to intramuscular, intradermal, intranasal, or oral, according to conventional protocols. Reagents used for diagnosis of HPV infections and associated neoplasia may be formulated as type-specific products capable of detecting antibodies for one or more genotypes of HPV.

5.7. Characterization of VLP In Final Bulk Products

[00158] Immunological identification of recombinant products made according to the present invention is afforded by, for example, Western blot analysis using polyclonal sera for HPV L1 capsid antigens (linear epitopes) or by enzyme linked immunoadsorbent assay (ELISA) using monoclonal antisera for PV L1 conformational epitopes specific for neutralizing antibodies. For Western blot analyses, aliquots (2 μg) of recombinant proteins from crude lysates, purified intermediates, or purified VLPs and control L1 capsid proteins are heat denatured (5-10 min. at 95-99°C) under reduced conditions with β-mercaptoethanol (10 mM) and loaded onto 4-12% NuPAGE (Novex) protein gels (FIG. 9) or equivalent polyacrylamide gels.

[00159] Proteins are resolved by gel electrophoresis in MES buffer under reduced conditions. Control proteins include recombinant PV L1 capsid proteins verified for authenticity, host cell proteins, and/or *Ac*MNPV baculovirus proteins. Protein molecular weight markers are, for example, SeeBlue pre-stained standards (Novex) including proteins with molecular weights of 188 kilodaltons (kD), 62 kD, 49 kD, 38 kD, 28 kD, 18 kD, 14 kD, 6 kD, and 3 kD. For protein gels, the electrophoresced proteins are visualized by staining with Colloidal Coomassie Blue reagent (Novex). The molecular weights of the L1 proteins are 50 – 65 kD depending on the species and genotype of papillomavirus capsid gene. The purity of purified recombinant HPV L1 VLPs purified by the invention is expected to be 95% or more as

determined by scanning densitometry. No more than 5% of the purified recombinant HPV L1 VLP product is expected to be proteolytic breakdown products.

[00160] For Western blot analysis (**FIGs. 10A - 10C,** as also described with reference to Example 21, below), proteins are transferred by electroblotting in methanol from unstained protein gels containing L1 capsid proteins and control proteins to nitrocellulose or polyvinyldifluoride membranes. Bound membranes are reacted with primary antisera including antisera to PV L1 capsid proteins, polyclonal sera to host cell proteins, and/or polyclonal sera #3 to *Ac*MNPV wild type baculovirus proteins. Bound primary antibodies are reacted with secondary antisera comprised of anti-IgG conjugated to alkaline phosphatase. The bound secondary antibodies are detected by reacting with the chromogenic substrate such as NBT/BCIP (InVitrogen) or the chemiluminescent substrate Lumi-Phos (In Vitrogen). The anti (α)-papillomavirus L1 sera is expected to detect protein bands with molecular weights of about 50 to 65 kD depending on the species and genotype of papillomavirus L1 capsid gene. Less than 5% of the recombinant PV L1 VLP products purified by the invention is expected to be degradation breakdown products. Less than 5% reactivity is expected to be seen using antisera to host or vector proteins.

[00161] The potency of recombinant HPV VLPs according to the present invention are ascertained by, for example, ELISA testing using antibodies specific for conformational epitopes on papillomavirus L1 capsid proteins that elicit neutralizing antibodies. In one embodiment of the present invention, ELISA testing of recombinant HPV-16 L1 VLP bulk products is performed using murine monoclonal antibody H16.V5 (Christensen *et al.*, 1996). Dilutions of VLPs (antigen) and control proteins such as VLPs, denatured VLPs, and heterologous proteins

are bound to wells of ELISA plates, and a constant amount of monoclonal antibody is added to each well.

[00162] Antigen-antibody binding occurs for at least two time durations, such as one minute and 2.5 hours. Antigen-antibody complexes are washed successively with wash buffer to remove nonspecific antigens. A secondary antibody comprised of anti-murine immunoglobulins conjugated to an enzyme such as horseradish peroxidase is added to each well of the ELISA plate. Detection of antigen-antibody complexes is afforded by the addition of a chromogenic substrate such as NBT/BCIP (InVitrogen).

[00163] As depicted in FIG. 11, and also described with reference to Example 20, below, the L1 proteins of Triton-treated recombinant HPV VLPs made according to the methods of the present invention are not degraded, as determined by SDS-PAGE and Western blot analyses. Triton-treated recombinant HPV VLPs remain as intact VLPs, as determined by analytical size exclusion chromatography and/or electron microscopy. The conformational epitopes of Triton-treated recombinant HPV VLPs made according to the methods of the present invention are restored by diafiltration against 0.5 M sodium chloride buffers, as determined by ELISA analysis using monoclonal antibodies raised against neutralizing epitopes of L1 antigens such as H16.V5 monoclonal antibody.

[00164] The amount of total protein purified by the methods of the present invention are determined by one of several colorimetric methods such as the bicinchoninic acid (BCA) assay or other protein quantitation assay by one skilled in the art of protein chemistry. The absolute amount of protein are determined by acid hydrolysis and amino acid determination. The results are compared with those results from colorimetric assays to adjust the relative amounts.

[00165] The amount of intact VLPs present following purification of recombinant HPV VLPs according to the present invention are ascertained by analytical size exclusion chromatography and/or electron microscopy. Size exclusion chromatography (SEC) are used to assess the relative amount of VLPs in production lots of HPV VLPs and and the relative amount of other viral VLPs made according to the present invention. In one embodiment, the pre-poured column used for SEC HPLC is an analytical size-exclusion HPLC column such as a TSK-GEL G6000PWXL column (Tosoh Biosep) that is used with, for example, a fractionation range of more than 1,000,000 daltons to approximately 20,000 daltons. The test sample is applied to the column with a resolution for intact VLPs at approximately 15-16 minutes and monomeric proteins at approximately 24-26 minutes.

[00166] Other macromolecules such as capsomeres of pentameric HPV L1 structures are resolved at 19-20 minutes when present. An analytical HPLC system such as a Waters 6000 HPLC system using Millennium computer software provides the mechanics and programs necessary for sample injection, buffer transfer, column development, UV monitoring, fraction collection, and protein data management. Data is presented in a graphic format with protein absorbance as a function of column development in minutes, as exemplified by FIG. 12 and also described with reference to Example 20, below. Confirmation of SEC HPLC results on viral VLPs is obtained by negative-stain electron microscopy (EM). Purified recombinant HPV VLPs are adsorbed onto carbon coated transmission electron microscopy (TEM) grids, stained with 1% uranyl acetate, and examined with a Philips electron microscope at 36,000x magnification. Results are shown in FIG. 13, also described with reference to Example 20, below. The size of the HPV-16 L1 VLPs is estimated about 40–55 nm.

6. *Pharmaceutical Compositions*

[00167] The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of one or more recombinant viral gene products, VLPs, agonists, antagonists, or a biologically active fragment of a viral gene product. The recombinant papillomavirus gene products preferably comprise HPV VLPs. More preferably, VLPs are HPV L1 VLPs, or chimeric VLPs. Administration of the pharmaceutical compositions of the invention, including vaccines, results in a detectable change in the physiology of a recipient subject, preferably by enhancing a humoral or cellular immune response to one or more papillomavirus antigens.

[00168] A multivalent vaccine of the present invention can confer protection to one or more genotypes of papillomavirus. The present invention thus concerns and provides a means for preventing or attenuating infection by at least one papillomavirus genotype. As used herein, a vaccine is said to prevent or attenuate a disease if its administration to an individual results either in the total or partial attenuation (*i.e*,. suppression) of a symptom or condition of the disease, or in the total or partial immunity of the individual to the disease.

[00169] The "protection" provided need not be absolute, *i.e.*, the papillomavirus infection need not be totally prevented or eradicated, provided that there is a statistically significant improvement relative to a control population. Protection can be limited to mitigating the severity or rapidity of onset of symptoms of the disease.

[00170] The pharmaceutical preparations of the present invention, suitable for inoculation or for parenteral or oral administration, are in the form of sterile aqueous or non-aqueous

solutions, suspensions, or emulsions, and can also contain auxiliary agents or excipients that are known in the art. The pharmaceutical composition of the invention can further comprise immunomodulators such as cytokines which accentuate the immune response. (*See, i.e.*, Berkow *et al., eds:.The Merck Manual,* Fifteenth Edition, Merck and Co., Rahway, N.J., 1987; Goodman *et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics,* Eighth Edition, Pergamon Press, Inc., Elmsford, N.Y., 1990; *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics,* Third Edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, Md., 1987; and Katzung, *ed. Basic and Clinical Pharmacology,* Fifth Edition, Appleton and Lange, Norwalk, Conn., 1992, which references and references cited therein, are entirely incorporated herein by reference as they show the state of the art.

[00171] As would be understood by one of ordinary skill in the art, when a composition of the present invention is provided to an individual, it can further comprise at least one of salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. Adjuvants are substances that can be used to specifically augment at least one immune response. Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately.

[00172] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous

dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

[00173] Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, mannitol, sorbitol, trehelose, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

[00174] The pharmaceutical composition of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[00175] Adjuvants can be generally divided into several groups based upon their composition. These groups include lipid micelles, oil adjuvants, mineral salts (for example, $AlK(SO_4)_2$, $AlNa (SO_4)_2$, $AlNH_4 (SO_4)$), silica, kaolin, polynucleotides (for example, poly IC and poly AU nucleic acids), and certain natural substances, for example, wax D from *Mycobacterium tuberculosis*, substances found in *Corynebacterium parvum*, or *Bordetella pertussis*. Preferred adjuvant of the invention includes, for example, Freund's adjuvant (DIFCO), alum adjuvant (Alhydrogel), MF-50 (Chiron) Novasomes™, or micelles, among others.

[00176] A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically or prophylactically effective amount" if the amount administered is physiologically significant.

[00177] The pharmaceutical composition of the invention is administration through various routes, including , subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Subcutaneous administration is preferred. Parenteral administration are achieved, for example, by bolus injection or by gradual perfusion over time.

[00178] A typical regimen for preventing, suppressing, or treating a disease or condition which can be alleviated by a cellular immune response by active specific cellular immunotherapy, comprises administration of an effective amount of the composition as described above, administered as a single treatment, or repeated as enhancing or booster dosages, over a period up to and including one week to about 48 months.

[00179] According to the present invention, an "effective amount" of a composition is an amount sufficient to achieve a desired biological effect, in this case at least one of cellular or humoral immune response to a papillomavirus genotype. It is understood that the effective dosage will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation.

[00180] This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims. The contents of all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

EXAMPLE 1

ESTABLISHMENT OF SERUM-FREE SF-9 INSECT CELL LINE

[00181] A new insect cell line designated Sf-9S was derived from the parent *S. frugiperda* Sf-9 cell line (ATCC CRL-1771) by several rounds of selective processes based on serum-independent growth and enhanced expression of secreted recombinant proteins from baculovirus vectors. Specifically, Sf-9 cells were cultivated to passage 38 in Grace's insect media (Life Technologies, Grand Island, NY 14072) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY 14072) as monolayer cultures in T-75 flasks (Corning, Inc., Corning, NY). The master cell bank of Sf-9 cells was stored at passage 38 in serum-containing media at –70°C and in liquid nitrogen. A working cell bank was established from a single cryovial of the Sf-9 master cell bank and cultivated in serum-containing insect media for an additional five (5) passages.

[00182] Initially, cell clones capable of growing in commercial serum-free media as suspension cultures were isolated from monolayer cultures of parent Sf-9 cells dependent on serum-containing media by sequential weaning of parent cells from serum-containing media. This process involved the plating of cell aliquots (200 μl) from a cell suspension (one cell per 200 μl) of the parent cell line in serum-containing media onto 96-well dishes at a ratio of 200 μl per well. Following attachment of cells and inspection of wells for wells with more than one cell, the media was changed from serum-containing media (100%) to a media mixture comprised of 75% serum-containing media and 25% serum-free media. After one to two weeks in culture, the media was changed from wells that initially contained only one cell per well and demonstrated cell growth and replication (*i.e.* four to five cells).

[00183] The second media mixture was comprised of 50% serum-containing media and 50% serum-free media. The cells were allowed to grow for another one to two weeks. The media was changed from wells containing cells that continued to grow and replicate. The third media mixture was comprised of 25% serum-containing media and 75% serum-free media. The cells were allowed to grow and replicate. After another two to four weeks, the media was changed from wells containing cells that continued to grow and replicate. The final media was comprised of serum-free media (100%). During each round of the weaning process, more than 90% of the cells did not survive the reduction in serum. This high level of cell death created a selective pressure to permit development of a new cell phenotype. Cells from wells that demonstrated continuous cell growth and replication were harvested and seeded into larger culture vessels. When a total cell density of > 4 x 10^6 cells was obtained, cells were seeded into shaker flasks (50 ml) as 10 ml suspension cultures with a starting cell density of 0. 2 - 0.5 x 10^6

cells/ml. Eight (8) clones that grew exponentially to a saturation cell density of > 6 x 10^6 cells/ml in serum-free media was selected, expanded, and frozen. One of the clones was established as a serum-free independent cell line.

EXAMPLE 2

ESTABLISHMENT OF TRANSFORMED SF-9S CELL LINE

[00184] In a second selection process, one of the serum-free cell clones developed in Example 1 was chosen to select cell clones that may produce enhanced levels of recombinant extracellular proteins and VLPs from several viruses including rotaviruses and human papillomaviruses by successive rounds of clonal selection of cells infected with recombinant baculoviruses and expressing extracellular self-assembled VLPs.

[00185] This process involved the plating of cell aliquots (200 µl) from a cell suspension (one cell per 200 µl) of the parent cell clone (#23) in serum-free media onto 96-well dishes at a ratio of 200 µl per well. From wells containing a single cell in the original seeding, cells were grown to confluency and subcultured into six replica-plates (96-well). The first round of selection was performed when a total cell density of 2 - 4 x 10^3 cells/well was obtained; the cells were infected with recombinant baculoviruses encoding human rotavirus virus VP2 and VP6 capsid genes. After three days of baculovirus infection, the infected cells and extracellular media were harvested by centrifugation. Infected cells were solubilized by adding 250 µl of 1% sodium dodecyl sulfate (SDS) and 10 mM β-mercaptoethanol (β-ME). SDS and β-ME were added to extracellular supernatants to final concentrations of 1% and 10 mM, respectively. Aliquots (10 µl) of solubilized cell lysates and extracellular media were heat-denatured (99°C for

10 min.) under reduced conditions and analyzed by SDS-PAGE and Western blotting using antisera to rotaviruses.

[00186] After review of the test results from the first virus infection, twenty four (24) cell clones demonstrating the highest levels of extracellular recombinant rotavirus VLPs were identified, seeded into 96-well plates, grown to confluency, and infected with a second recombinant baculovirus encoding HPV-16 L1 capsid protein. At three days post-infection, infected cells and extracellular supernatants were produced by centrifugation of infected cell suspensions from the plate.

[00187] Infected cells and extracellular supernatants were analyzed by SDS-PAGE and Western blot analyses using polyclonal anti-HPV-16 L1 sera. The test results of both viral infections were reviewed and compared. One cell clone (#12) that produced the high levels of extracellular VLPs from rotavirus and HPV capsid proteins was chosen to establish a cell line capable of producing extracellular VLPs. To establish a cell line from the selected cells, cells from an uninfected replica plate were amplified at 28°C and 150 rpm in a platform shaker incubator into a suspension culture using Sf-900 II serum free media (GIBCO). The amplified cell culture was diluted to a seeding cell density of 0.25 x 10^6 cells/ml, grown in 100 ml of Sf-900 II SFM within a 500 ml shaker flask, and subcultured at a split ratio of 1:20 for forty three passages. After continuous passaging, the cell line was established and was passaged three more times to establish a master cell bank.

EXAMPLE 3

CLONING CODON-OPTIMIZED HPV-16 L1 GENES AND ESTABLISHMENT OF RECOMBINANT BACULOVIRUS STOCKS

[00188] A HPV-16 L1 prototype (GenBank Accession No. K02718) and modified in U.S. Patent No. 5,985,610, was optimized for codon usage in insect cells of the Lepidopteran family. The HPV-16 L1 gene was optimized (FIG.1A) in this embodiment of the present invention for codon usage based on the following criteria: (1) abundance of aminoacyl-tRNAs for a particular codon in Lepidopteran species of insect cells for a given amino acid as described by Levin and Whittome (2000); (2) maintenance of GC-AT ratio in L1 gene sequence at approximately 1:1; (3) minimal introduction of palindromic or stem-loop DNA structures, and (4) minimal introduction of transcription and post-transcription repressor element sequences.

[00189] The optimized gene sequence was synthesized *in vitro* as overlapping oligonucleotides, cloned into a subcloning plasmid vector, and then cloned into a bacmid transfer vector (*i.e.*, Luckow *et al.*, 1993), according to procedures known in the art (*i.e.*, Summers and Smith, 1987). The bacmid transfer vector pFASCTBAC1 with HPV-16 L1 gene was used to transform competent *E. coli* DH10BAC cells and produce recombinant bacmid DNA. The recombinant bacmid DNA with the L1 gene was transfected into insect cells to produce recombinant baculoviruses encoding L1 genes.

[00190] In particular, a restriction fragment (*Bam* HI/*Sal* I restriction fragment (1572 bp) containing a HPV-16 L1 gene (K strain)) containing a HPV L1 capsid gene from a natural virus isolate or synthesized gene is ligated to a bacmid transfer vector, such as pFASTBAC-1 (*see,* for

example, Luckow *et al.*, 1993), at the multiple cloning site, which contains a Tn7 transposable element surrounded by the transcription promoter and polyadenylation/transcription termination elements of the polyhedrin (*polh*) gene from a wild type *Ac*MNPV genome. Competent *E. coli* DH10BAC cells, which contain bacmid DNA (an *Ac*MNPV baculovirus genome with a Tn7 transposable element within the polyhedrin locus), are transformed with the bacmid transfer vector containing the HPV L1 gene.

[00191] Recombinant bacmids are produced by site-directed recombination between the respective Tn7 transposable elements of the transfer vector and the bacmid genome resulting in the production of recombinant bacmid genomes encoding the optimized L1 gene in the *E. coli* hosts. The recombinant bacmid DNA is isolated for example by miniprep DNA isolation and transfected into Sf-9S insect cells to produce recombinant baculoviruses encoding the L1 genes.

[00192] The progeny recombinant baculoviruses (~ 10^4 plaque forming units) are plaque-purified (3x) and selected for high expression of the HPV-16 L1 gene product, as determined by SDS-PAGE and Western blot analyses using rabbit polyclonal antisera specific for the HPV16 L1 gene product (Pharmingen). A HPV-16 L1 master virus stock is prepared in Sf-9S insect cells, as described in Example 2, from one of the plaque-purified clones expressing high levels of recombinant HPV-16 L1 proteins that self-assemble into virus-like particles and is qualified for safety and biological properties as described below. Working virus stocks of HPV-16 L1-expressing baculoviruses are prepared by infection of Sf-9S insect cells at a multiplicity of infection of 0.1 pfu/cell with the qualified HPV-16 L1 master virus stock and are characterized as described below to qualify for recombinant HPV-16 L1 VLP product manufacturing.

EXAMPLE 4

CLONING CODON-OPTIMIZED HPV-16 CHIMERIC GENES AND ESTABLISHMENT OF CHIMERIC RECOMBINANT BACULOVIRUS STOCKS

[00193] HPV-16 L2 fusion genes are optimized for codon usage in insect cells as described above for L1 genes (FIG. 1C – 1E). The L2/E7/E2 fusion gene sequence is synthesized *in vitro* as overlapping oligonucleotides, cloned into a subcloning plasmid vector, and then cloned into a bacmid transfer vector according to procedures known in the art (*i.e.*, Luckow *et al.*, 1993; Summers and Smith, 1987).

[00194] For example, a baculovirus transfer vector with a L2 fusion gene is co-transfected into insect cells with linearized wild-type baculovirus genomic DNA to produce recombinant baculoviruses encoding L2 fusion genes. Alternatively, the bacmid transfer vector with the L2 fusion gene is used to transform competent *E. coli* DH10BAC cells and produce recombinant bacmid DNA. The codon-optimized gene is cloned into a bacmid transfer vector (*i.e.*, Luckow *et al.*, 1993).

[00195] In particular, a *Bam* HI/*Kpn* I restriction DNA fragment (2834 bp) containing a HPV-16 L2/E7/E2 fusion gene was ligated with T4 DNA ligase to a *Bam* HI/*Kpn* I digest of the bacmid transfer vector pFASTBAC-1 (Luckow *et al.*, 1993) at the multiple cloning site. Competent *E. coli* DH10BAC cells were transformed with the bacmid transfer vector containing the HPV-16 L2/E7/E2 fusion gene. Recombinant bacmids were produced by site-directed recombination between the respective Tn7 transposable elements of the transfer vector and the

bacmid genome resulting in the production of recombinant bacmid genomes encoding the optimized HPV L1 gene in the *E. coli* hosts.

[00196] The recombinant bacmid DNA was isolated by miniprep DNA isolation and transfected into Sf-9S insect cells to produce recombinant baculoviruses encoding the HPV-16 L2/E7/E2 L2 fusion genes. The recombinant baculoviruses were plaque-purified (3x) in Sf-9S insect cells and selected for high expression of the HPV-16 L2, E7, and E2 gene products, as determined by SDS-PAGE and Western blot analyses using antisera specific for each peptide within the L2 fusion gene product (HPV-16 L2, E7, and E2 peptides). A master virus stock of baculoviruses expressing HPV-16 L2/E7/E2 fusion proteins was prepared in Sf-9S insect cells from one of the plaque-purified clones expressing high levels of recombinant HPV L2 fusion proteins and was qualified for safety and biological properties. Working virus stocks of baculoviruses expressing HPV-16 L2/E7/E2 fusion proteins were prepared in Sf-9S insect cells at a multiplicity of infection of 0.1 pfu/cell with the qualified master virus stock and were tested.

EXAMPLE 5

CHARACTERIZATION AND QUALIFICATION OF L1 AND CHIMERIC RECOMBINANT BACULOVIRUS STOCKS

[00197] Recombinant baculovirus stocks for each of the HPV L1 and L2 fusion viruses were established. Master and working virus stocks were established from high expression virus clones and characterized for safety and biological properties. Safety properties of master and working virus stocks included microbial sterility, adventitious agent presence, endotoxin level, spiroplasma, and mycoplasma contaminants, and the like. Biological properties included genetic

identity, virus titer, viral replication competence, and recombinant protein production competence. The genetic identity of the master virus stock was determined, for example, by DNA sequence analysis of both strands of bacmid DNA encoding the HPV L1 or L2 fusion genes and flanking sequences. The virus titer of master and working virus stocks were determined by an agarose plaque assay using insect cells and serial dilutions of the virus stock.

[00198] Viral replication competency was evaluated by passage of an aliquot of the virus stocks in insect cells at low multiplicity of infection. Subsequent determination of the virus titer for the progeny virus passage was performed by agarose plaque assay. Recombinant protein expression competency was evaluated, for example, by infection of insect cells with an aliquot of the virus stocks and subsequent determination of the relative abundance of recombinant proteins such HPV L1 and L2 fusion proteins per total cell protein in infected cells by SDS-PAGE analysis.

EXAMPLE 6

VIRUS INFECTION FOR HPV-16 L1 VLPS

[00199] Recombinant HPV-16 L1 VLPs expressed in baculovirus-infected Sf-9S cells were purified from intracellular and extracellular crude lysates. Sf-9S insect cells from Example 2 were thawed from a single cryovial of the working cell bank frozen at -70°C in Sf-900 II SFM insect cell media (GIBCO) at a concentration of 1.0×10^7 cells/ml. Thawed cells were seeded into 50 ml of Sf-900 II SFM insect cell media and cultured as suspension cultures in 500 ml shaker flasks in a platform shaker incubator at 28^0C with an agitation speed of 125 rpm.

[00200] After the cell density reached 6 x 10^6 cells/ml and a cell viability of more than 95%, the culture was seeded into two (2) liter flasks in a final volume of 800 ml of insect serum-free media per flask at a starting seed density of 0.5 x 10^6 cells/ml. The cells were cultured in a platform shaker incubator at 28^0C with an agitation speed of 100 - 125 rpm. When the cell density reached 2 -3 x 10^6 cells/ml, the insect cells were infected with a recombinant baculovirus encoding the HPV-16 L1 capsid gene (K strain) from the *polh* locus made according to Example 3. The virus infection was established at a MOI of 3 pfu per cell. The virus infection was carried out for six days in a platform shaker incubator at 28^0C with an agitation speed of 125 rpm. The infected cells were harvested by centrifugation (at 1,500 × g and 2 - 8^0 C for 10 minutes) after the following conditions were met: cell viability was less than 25%, and L1 gene products were in culture fluids and within infected cells.

EXAMPLE 7

VIRUS INFECTION FOR HPV-16 CHIMERIC VLPS

[00201] Production of intracellular HPV-16 L2/E7/E2 chimeric VLPs began with high multiplicity infection of log phase Sf-9S insect cells (1.5 x 10^6 cells/ml) in suspension shaker flask cultures (2 L) containing serum-free insect cell medium (800 ml; HyQ SFM media, HyClone) made according to Example 2 with aliquots of HPV-16 L1 and L2/E7/E2 working virus stocks prepared according to Example 4. The ratio of co-infecting viruses was approximately 1:10 (L1 to L2 virus). The virus infection was monitored daily by the trypan blue exclusion method for cytopathic effects, cell viability, and cell density and by SDS-PAGE and Western blot analyses of recombinant HPV L1 and L2 fusion proteins in infected cells. At three

days post-infection when peak recombinant HPV-16 L1 and L2/E7/E2 fusion gene expression occurred, infected cells containing intracellular HPV-16 chimeric VLPs were harvested by centrifugation at 1500 x g and 2-8°C for 5 minutes and processed to obtain purified HPV-16 chimeric VLP products.

EXAMPLE 8

PREPARATION OF CRUDE CELL LYSATES FOR HPV-16 CHIMERIC VLPS

[00202] Recombinant HPV-16 chimeric VLPs including recombinant HPV-16 L2/E7/E2 fusion proteins encapsulated into HPV-16 L1 VLPs produced according to Example 6 were harvested from modified Sf-9 insect cells infected with recombinant baculoviruses encoding the HPV-16 L1 capsid gene (K strain) and HPV-16 L2/E7/E2 fusion gene. Infected cells were harvested by low-speed centrifugation at 1,500 x g and 2 - 8°C for 10 minutes. Infected cell pellets containing intracellular recombinant HPV-16 chimeric VLPs were resuspended in phosphate-buffered saline II solution (1.54 mM KH_2PO_4, 2.71 mM $Na_2HPO_4 \bullet 7H_2O$, and 154 mM NaCl (pH 7.2)) at a ratio of 10 ml buffer per gram of cell pellet. Protease inhibitors corresponding to serine, cysteine, and aspartate classes of proteases were added to the following final concentrations: (PMSF, 1 mM; Aprotinin, 1 μg/ml; Leupeptin, 10 μg/ml; Pepstatin, 5 μg/ml). The resuspended cells containing intracellular recombinant papillomavirus VLPs were disrupted by mild sonication in phosphate-buffered saline solution with two (2) pulses at 200-300 watts and 2 – 8°C with a Branson Model 250 sonifier equipped with a 1/8" probe. The result of sonication was a crude cell lysate containing intact intracellular recombinant HPV-16 chimeric VLPs with minimal disruption of cellular proteosomes and degradation of HPV-16 L2/E7/E2 fusion proteins.

EXAMPLE 9

Clarification of HPV-16 L1 VLP Crude Cell Lysates and Supernatants

[00203] Crude cell lysates made according to Example 7 containing intracellular recombinant HPV-16 L1 VLPs were clarified by centrifugation at 12,000 x g and 2 - 8°C for 60 minutes to remove large cellular debris and membranes. Clarified supernatants were collected by aspiration, and cellular pellets were discarded. Crude media supernatants containing extracellular recombinant HPV-16 L1 VLPs were clarified by centrifugation at 12,000 x g and 2 - 8°C for 60 minutes to remove large cellular debris and membranes. Clarified supernatants were collected by aspiration, and cellular pellets were discarded.

EXAMPLE 10

CLARIFICATION OF HPV-16 CHIMERIC VLP CRUDE CELL LYSATES

[00204] Crude cell lysates made according to Example 8 were purified as follows. The cell lysate containing intracellular recombinant HPV-16 chimeric VLPs harvested from insect cells infected with recombinant baculoviruses encoding HPV-16 L1 and L2/E7/E2 fusion proteins were clarified by centrifugation at 12,000 x g and 2 - 8°C for 60 minutes to remove large cellular debris and membranes. Clarified supernatants were collected by aspiration, and cellular pellets were discarded.

EXAMPLE 11

Concentration and Diafiltration of HPV-16 VLP Clarified Supernatants

[00205] Concentration and diafiltration steps of the present invention involved ultrafiltration of clarified supernatants made according to Example 9. The clarified supernatant

contained intracellular and extracellular recombinant HPV-16 L1 VLPs. These VLPs were expressed from infection of Sf-9S insect cells with recombinant baculovirus encoding the L1 capsid gene of HPV-16 (K strain). Clarified supernatants containing intracellular and extracellular recombinant HPV-16 L1 VLPs were concentrated ten fold by ultrafiltration using an Amicon M-12 Proflux Tangential Flow Ultrafiltration System equipped with a hollow fiber ultrafiltration cartridge (A/G Technologies Model UFP-500-C-55A). Concentrates containing intracellular and extracellular recombinant HPV-16 L1 VLPs were diafiltered against eight volumes of cation exchange loading buffer solution containing 20 mM sodium phosphate (pH 5.7) and 10 mM sodium chloride by ultrafiltration using an Amicon M-12 Proflux Tangential Flow Ultrafiltration System equipped with a hollow fiber ultrafiltration cartridge (A/G Technologies Model UFP-500-C-55A) at an initial flow rate of 0.8 L/min. and an inlet/outlet pressure of 8 psi.

EXAMPLE 12

CATION EXCHANGE CHROMATOGRAPHY OF HPV-16 VLP CONCENTRATED DIALYSATES

[00206] Diafiltered concentrates containing intracellular and extracellular recombinant HPV-16 VLPs made according to Example 11 were loaded onto a chromatography column containing Streamline SP Adsorptive resin (Amersham Biosciences), a strong cation exchange chromatography resin, at a flow rate of 0.5 L/hr and a ratio of 1 ml resin per 1 gram of diafiltrate. The chromatographic column was developed with a Waters 6000 HPLC System. The SP resin was equilibrated with a cation binding buffer (50 mM sodium phosphate (pH 5.7) and 10 mM sodium chloride). Following binding of diafiltrates containing intracellular and extracellular recombinant HPV-16 VLPs, the column was rinsed with five (5) volumes of cation binding

buffer. Bound proteins were eluted as 1 ml fractions with UV-monitoring at 214 nm from the column resin at a flow rate of 0.5 L/hr using a step pH gradient from 6.0 to 8.0 in 20 mM sodium phosphate with 10 mM sodium chloride. Fractions containing intracellular and extracellular recombinant HPV-16 VLPs eluted in the 7.0 - 7.5 steps, as determined by SDS-PAGE and Western blot analysis of elution fraction samples using antisera against papillomavirus L1 capsid proteins. Those fractions containing HPV-16 L1 VLPs were pooled.

EXAMPLE 13

AFFINITY CHROMATOGRAPHY OF HPV-16 VLP CATION EXCHANGE CHROMATOGRAPHY ELUATES

[00207] Pooled eluates from the cation exchange chromatography that contain intracellular and extracellular recombinant HPV-16 L1 VLPs made according to Example 12 were dialyzed against 100 volumes of affinity loading buffer (20 mM sodium phosphate (pH 5.7), 2 mM EGTA, and 300 mM sodium chloride) for 8 - 16 hrs. The dialyzed material was loaded onto a column containing heparin agarose (Amersham Biosciences) at a flow rate of 1 ml/min. The ratio of packed heparin agarose to protein was 1 ml of resin per 0.5 grams of protein. Bound proteins including recombinant HPV-16 L1 VLPs were eluted as 1 ml fractions with UV-monitoring at 214 nm from the column resin at a flow rate of 1 ml/min. using a linear salt gradient from 300 mM to 2 M. Fractions containing recombinant HPV-16 L1 VLPs eluted in salt fractions from 500 - 700 mM, as determined by SDS-PAGE and Western blot analysis of elution fraction samples using antisera against papillomavirus L1 capsid proteins. Those fractions containing HPV-16 L1 VLPs were pooled.

EXAMPLE 14

ALTERNATIVE AFFINITY CHROMATOGRAPHY OF HPV-16 VLP CATION EXCHANGE CHROMATOGRAPHY ELUATES

[00208] Pooled eluates from cation exchange chromatography that contain intracellular and extracellular recombinant HPV-16 L1 VLPs made according to Example 12 were dialyzed against 100 volumes of affinity loading buffer (20 mM sodium phosphate (pH 5.7), 2 mM EGTA, and 300 mM sodium chloride) for 8 - 16 hrs. The dialyzed material was loaded onto a column containing Matrex Cellufine Sulfate (Amersham Biosciences) at a flow rate of 1 ml/min. The ratio of packed Matrex Cellufine Sulfate to protein was 1 ml of resin per 0.5 grams of protein. Bound proteins including recombinant HPV-16 L1 VLPs were eluted as 1 ml fractions with UV-monitoring at 214 nm from the column resin at a flow rate of 1 ml/min using a linear salt gradient from 300 mM to 2 M. Fractions containing recombinant HPV-16 L1 VLPs eluted in salt fractions from 400 mM to 600 mM, as determined by SDS-PAGE and Western blot analyses of elution fraction samples using antisera against papillomavirus L1 capsid proteins. Those fractions containing HPV-16 L1 VLPs were pooled.

EXAMPLE 15

ANION DISPLACEMENT CHROMATOGRAPHY OF HPV-16 VLP AFFINITY CHROMATOGRAPHY ELUATES

[00209] Pooled eluates from affinity chromatography that contain recombinant HPV-16 L1 VLPs made according to Examples 13 or 14 were dialyzed against 100 volumes of anion loading buffer (0.24 M Tris-HCl (pH 8.0)) for 8 - 16 hrs. The dialyzed material was loaded onto a column containing Q Sepharose FF (Amersham Biosciences), a strong anion exchange

chromatography resin, at a flow rate of 0.5 ml/min. The ratio of packed Q Sepharose to protein was 1 ml of resin per 0.1 gram of protein. Bound proteins including recombinant papillomavirus VLPs were displaced as 1 ml fractions with UV-monitoring at 214 nm from the column resin at a flow rate of 0.5 ml/min using a linear gradient from 0 to 5 mg/ml dextran sulfate (5000 MW). Fractions containing recombinant HPV-16 L1 VLPs eluted in dextran sulfate fractions from 4 mg/ml to 5 mg/ml, as determined by SDS-PAGE and Western blot analyses of elution fraction samples using antisera against papillomavirus L1 capsid proteins. Those fractions containing HPV-16 L1 VLPs were pooled. Pooled eluates from anion exchange chromatography that contain recombinant papillomavirus VLPs were dialyzed against 100 - 150 volumes of final bulk storage buffer (5 mM $Na_2HPO_4 \bullet 7H_2O$, 5 mM KH_2PO_4, and 500 mM NaCl (pH 6.8)) for 8 - 16 hrs.

EXAMPLE 16

LINEAR SUCROSE GRADIENT PURIFICATION AS AN ALTERNATIVE TO L1 VLP CHROMATOGRAPHIC VLP PURIFICATION

[00210] Intracellular and extracellular HPV VLPs were also purified from concentrated crude cell lysates and media supernatants made according to Examples 9 or 10 by ultracentrifugation on linear sucrose gradients. Concentrates (5 – 10 g) containing HPV L1 VLPs were loaded at a flow rate of 100 – 250 ml per minute onto 0 –65% linear sucrose gradient prepared in phosphate-buffered saline solution (5 mM potassium phosphate (monobasic), 5 mM sodium phosphate (dibasic), 154 mM sodium chloride (pH 7.2)) in a RK-2 vertical rotor (1.6 L) accelerating at 35,000 rpm in a RK continuous flow ultracentrifuge (Schiaparelli Instruments, Amsterdam, The Netherlands). The gradient was resolved by centrifugation at 35,000 rpm and

15 – 25°C for one to two hours residence and one hour coasting to a complete stop without braking. Gradient material from the sucrose gradient was passed through a UV_{218nm} monitor and collected as 50 ml aliquots in a fraction collector. Samples from each fraction were subjected to SDS-PAGE and Western blot analyses to find HPV-16 L1 VLPs. Results from these analyses indicated that recombinant HPV-16 L1 VLPs sedimented into two bands corresponding to 43–53% sucrose and 30 – 40% sucrose. The baculoviruses sedimented as one band corresponding to 30-35% sucrose. Fractions 6 –9 containing recombinant HPV-16 L1 VLPs comprised of HPV-16 L1 protein species with molecular weights of 55 and 60 kD were pooled. Fractions 10 – 14, which contained recombinant HPV-16 L1 proteins with molecular weights of 50 and 55 kD and proteolytic breakdown products of L1 proteins, were not pooled and used as product due to the proteolysis. The pooled L1 VLP fractions were diluted 6 fold with the PBS solution, and subjected to a second round of ultracentrifugation on linear sucrose gradients, except the second gradient was 0-50% sucrose (PBS) and was run for a total of two hours (one hour residence and one hour coasting). Sucrose gradient fractions from 0-50% linear sucrose gradients were examined for HPV-16 L1 VLPs by SDS-PAGE, SEC HPLC, Western blot, and ELISA analyses. Fractions containing intact HPV VLPs displaying conformational epitopes were pooled.

[00211] Pooled sucrose gradient fractions containing extracellular recombinant HPV-16 L1 VLPs were diafiltered by ultrafiltration against eight volumes of final VLP storage buffer containing 5 mM $Na_2HPO_4 \bullet 7H_2O$, 5 mM KH_2PO_4, and 500 mM NaCl (pH 6.8) using an Amicon M-12 Proflux Tangential Flow Ultrafiltration System equipped with a hollow fiber ultrafiltration cartridge (A/G Technologies Model UFP-500-C-55A) at an initial flow rate of 0.8 L/min and an inlet/outlet pressure of 8 psi. Triton X-100, a nonionic detergent and surfactant,

was added at a final concentration of 0.1% to the bulk HPV VLP product, which was incubated for two hours at ambient temperature to inactivate residual baculoviruses. Alternatively, the bulk HPV VLP product was irradiated with at least three rounds of ultraviolet (UV) light at 254 nm to inactivate residual baculoviruses. The treated material was filtered aseptically through a 0.22 µm membrane into silanized borosilicate glass containers.

EXAMPLE 17

SUCROSE STEP GRADIENT PURIFICATION OF HPV-16 L1 VLPS AS AN ALTERNATIVE TO L1 VLP CHROMATOGRAPHIC VLP PURIFICATION

[00212] In yet another alternate embodiment of the present invention, diafiltered concentrates made according to Examples 9 and containing HPV-16 L1VLPs were purified by rate-zonal ultracentrifugation on discontinuous sucrose step gradients. Diafiltrates containing recombinant HPV-16 L1VLPs were loaded onto approximately 25% sucrose cushions (prepared in PBS solution) in a swinging bucket rotor (Sorval Model AH 628) accelerating at 35,000 rpm and 2-8°C in an ultracentrifuge (Sorval Model OTB-65B) for three hours. The pellets at the bottom of the sucrose cushion were collected, while the sucrose cushion and load material were discarded. The sucrose cushion pellets were solubilized in PBS solution at approximately 1 g of pellet per ml of buffer 1 and loaded onto sucrose step gradients containing six steps comprising approximately 25, 30, 35, 40, 45, 50 and 65% sucrose. The sucrose step gradients were resolved by ultracentrifugation in a swinging bucket rotor at 35,000 and 2-8°C for 1 hour until recombinant HPV VLPs separate from baculovirus particles.

[00213] Gradient material from the first round of sucrose step gradients were monitored by ultraviolet light during collection in a fraction collector. Gradient fractions were analyzed by SDS-PAGE and Western blot analysis using antisera against HPV-16 L1 capsid proteins. Peak fractions containing HPV VLPs or their component proteins were pooled, diluted multifold with buffer solution, and were subjected to a second round of ultracentrifugation on sucrose step sucrose gradients. Gradient material from the second round of sucrose step gradients were monitored by ultraviolet light during collection in a fraction collector. Gradient fractions from the second round of ultracentrifugation were analyzed by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins. Peak fractions containing HPV-16 proteins were pooled. The purified recombinant HPV-16 L1 VLPs in the pooled fractions were formulated as recombinant HPV-16 L1 VLP products.

EXAMPLE 18

SUCROSE STEP GRADIENT PURIFICATION OF HPV-16 CHIMERIC L1 VLPS

[00214] In yet another alternate embodiment of the present invention, diafiltered concentrates made according to Example 10 and containing HPV-16 chimeric VLPs were purified by rate-zonal ultracentrifugation on discontinuous sucrose step gradients based primarily on mass rather density of recombinant HPV-16 chimeric VLPs in sucrose.

[00215] Diafiltrates containing recombinant HPV-16 chimeric VLPs were loaded onto approximately 25% sucrose cushions in a swinging bucket rotor accelerating at 35,000 rpm in an ultracentrifuge (Sorval) for three hours. The pellets at the bottom of the sucrose cushion were collected, while the sucrose cushion and load material were discarded. The sucrose cushion

pellets were solubilized in PBS buffer and loaded onto sucrose step gradients containing six steps comprising approximately 25, 30, 35, 40, 45, 50 and 65% sucrose.

[00216] The sucrose step gradients were resolved by ultracentrifugation in a swinging bucket rotor at 35,000 for 1 hour until recombinant HPV VLPs were separated from baculovirus particles. Gradient material from the first round of sucrose step gradients was monitored by ultraviolet light during collection in a fraction collector. Gradient fractions were analyzed by SDS-PAGE and Western blot analysis using antisera against HPV-16 L1 capsid proteins and L2, E7, and E2 fusion proteins. Peak fractions containing HPV VLPs or their component proteins were pooled, diluted multifold with buffer solution, and were subjected to a second round of ultracentrifugation on sucrose step sucrose gradients.

[00217] Gradient material from the second round of sucrose step gradients were monitored by ultraviolet light during collection in a fraction collector. Gradient fractions from the second round of ultracentrifugation were analyzed by SDS-PAGE and Western blot analysis using antisera against papillomavirus L1 capsid proteins and/or L2 fusion proteins. Peak fractions containing HPV-16 proteins were pooled. The purified recombinant HPV-16 chimeric VLPs in the pooled fractions were formulated as recombinant HPV-16 chimeric VLP products.

EXAMPLE 19

FORMULATION OF FINAL BULK HPV-16 L1 VLP PRODUCTS

[00218] Residual baculovirus present in recombinant HPV-16 L1 VLPs made according to Examples 15, 16, or 17 was inactivated by treatment of crude, intermediate, and final bulk products with the non-ionic detergent and surfactant, Triton X-100. Inactivation of virus in

HPV-16 L1 VLP products was provided by addition of Triton X-100 at a final concentration 0.1% for 2 hours at 15– 25°C. Following treatment, HPV VLP products were diafiltered against 4 x 1000 volumes of high salt buffer containing (5 mM $Na_2HPO_4 \bullet 7H_2O$, 5 mM KH_2PO_4, and 500 mM NaCl (pH 6.8)) and 2–8°C for 12 hours. Diafiltrates containing treated recombinant HPV-16 L1 VLPs were filtered aseptically through a 0.22 µm membrane at 15 – 25°C. Filtered HPV-16 L1 VLP final bulk products were dispensed into ten liter containers including 316L stainless steel tanks, silanized borosilicate glass bottles, and polyethylene plastic bioprocess bags and stored at 2 – 8°C for < six (6) months or at < -70°C for < 2 years.

EXAMPLE 20

ANALYSIS OF INTACT HPV VLPS

[00219] ELISA testing of solutions containing recombinant HPV-16 L1 VLPs (lot 1274), sucrose, and/or clarified supernatants from bHPV-16 L1 infected Sf-9S insect cells mock treated or treated two hours at room temperature with Triton X-100 (0.1% final concentration) was performed. Mock and treated solutions containing recombinant HPV-16 L1 VLPs were dialyzed against four changes of 1000 volumes of phosphate buffer containing 0.5 M sodium chloride. Various amounts (250, 500, and 1000 ng) of dialysates were bound to 96-well plates and reacted with antibodies from the murine hybridoma cell line H16.V5 that bind to HPV-16 L1 protein conformational epitopes that elicit neutralizing antibodies to determine the effect of Triton X-100 on these epitopes. The results as shown in FIG. 11 demonstrated that Triton had little or no effect on recombinant HPV-16 L1 VLPs with or without sucrose, but five fold decrease in binding activity was noted with 1000 ng of VLPs mixed with supernatants as compared to that of mock treated and Triton-treated VLPs or VLPs in sucrose. These results indicated that Triton

treatment of recombinant VLPs, which effectively afforded as much as 4 to 7 log reduction in baculovirus titers, did not irreversibly destroy conformational L1 epitopes of recombinant HPV-16 L1 VLPs in buffer or buffer with sucrose. However, VLPs amidst infected extracellular materials did not retain the proper epitope conformation after Triton treatment. The conclusion: the best time to add Triton in the purification of recombinant HPV VLPs was after downstream processing but prior to terminal filtration of final bulk products.

[00220] In one set of analyses as depicted in FIG. 12, samples (5 μg of phosphate buffered saline) from different lots (1207, 1244, 1265, and 1268) of recombinant HPV-16 L1 VLPs were injected to an analytical size exclusion column and resolved according to their mass and shape. The pre-poured column used for analytical size exclusion chromatographic analysis was a 15 cm TSK 6000PWXL stainless steel column (Tosohaus). The fractionation range of this column was more than 1,000,000 to 20,000 daltons. The volume of test sample injected into the column was 50 μl. A Waters 6000 HPLC system using Millennium computer software provided the automated mechanics and programs necessary for sample injection, buffer transfer, column development, UV monitoring, fraction collection, and protein data management.

[00221] As the result of the analysis, control blue dextran beads having a molecular weight of 2,000,000 daltons was resolved as a single peak at 10 – 12 minutes. The expected resolution of VLPs was at 15-16 minutes and monomeric proteins at 24-26 minutes. Pentameric HPV-16 L1 structures were expected to resolve at 19-20 minutes when present. Analysis of a final container vaccine vial production lot 1207 stored at –70°C for more than two years indicated that the recombinant HPV-16 L1 VLPs resolved as two peaks (FIG. 12). The major peak was at 15 min. and a minor peak was at 25 min. Upon integration of the area beneath each

peak, these data indicated that at least 95% of the L1 protein in this production remained as VLPs after more than two years frozen at –70°C. Results of samples from bulk lots 1244, 11265, and 1268 of recombinant HPV-16 L1 VLP showed in FIG. 12 a single major peak at 15 min. Following integration of the peak areas, > 95% of this vaccine product remained as VLPs more than two years after production. As recombinant HPV-16 L1 VLPs dissociate, pentamers at 20 min and monomers at 25 min. appeared (data not shown). Interestingly, bovine papillomavirus (BPV) VLPs, a relative of HPV-16, behaved similarly in this assay. Hawaii virus (HV) VLPs, calicivirus self-assembled macromolecules, exhibited >99% intact VLPs.

[00222] An electron micrograph of HPV VLPs was obtained by negative staining of 10 μl aliquots of recombinant HPV-16 L1 VLPs (1 mg/ml in phosphate buffered saline) with uranyl acetate and observing at high magnification (36,000x) on a transmission electron microscope (Phillips). These results as depicted in FIG. 13 demonstrated intact and discrete virus-like particles with a size of 40 to 55 nm and a morphology similar to papillomavirus virions. These electron micrographs provided another example of confirmatory evidence of high quality HPV-16 L1. Further, the SEC HPLC assay was shown again to be an effective measurement tool for quantitation of VLPs.

EXAMPLE 21

PRODUCTION OF ANTISERA TO HOST CONTAMINANT PROTEINS

[00223] Antisera to insect cell and wild type baculovirus proteins were produced to detect contaminating host proteins in baculovirus-derived recombinant protein products. For antiserum against insect cell proteins, 800 ml suspension shaker cultures of Sf-9S and High Five insect

cells were grown to cell density of 2×10^6 cell per ml. The cultured cells were harvested by centrifugation at 500 x g and 2-8°C for five minutes. The cell pellets were resuspended in 10 ml of phosphate-buffered saline solution (5 mM sodium phosphate, dibasic, 5 mM potassium phosphate, monobasic, 154 mM sodium chloride (pH 7.2)). The resuspended cells were disrupted by sonication with two (2) pulses at 200-300 watts and 2 – 8°C with a Branson Model 250 sonifier equipped with a 1/8" probe to produce cell lysates. The sonicated cell lysates were clarified by centrifugation at 12,000 x g and 2 - 8°C for 60 minutes to remove large cellular debris and membranes. The clarified supernatants were retained for protein quantitation by the BCA method, and the pellets were discarded.

[00224] Aliquots of the clarified cell lysates were formulated into immunogens by emulsification of equal volumes of antigen and complete Freund's adjuvant (DIFCO) at an antigen concentration of 200 μg/ml. The immunogens were administered intramuscularly (primary) as two doses (50 μg/dose) into the hindquarters of New Zealand rabbits. At four weeks post-immunization, a second round of immunization (booster) occurred as before except that incomplete Freund's adjuvant was used. At eight weeks post-immunization, sera were isolated from blood withdrawn from immunized animals.

[00225] The antibody titers of the immunized sera were determined by Western blot analysis using nitrocellulose membranes containing proteins from Sf-9S and High Five insect cells, as well as control protein samples from baculovirus- and *E. coli*-derived recombinant protein products. Results using these antisera from Sf-9S insect cell proteins demonstrated that the antibodies specific for this cell line were present, as positive binding was observed in lanes of

blots containing Sf-9S and Sf-9S infected cell proteins but not in lanes containing purified recombinant proteins such as HPV-16 L1 proteins (FIG. 10B).

[00226] Similarly, antisera were produced in rabbits against antigens comprised of wild-type baculovirus proteins expressed in Sf-9S and High Five insect cells infected at an MOI of 3 pfu/cell with *Ac*MNPV wild-type baculovirus for three days to produce infected cell lysates. These antisera were utilized to demonstrate the presence of baculovirus contaminants in baculovirus-derived recombinant protein products (FIG. 10C). Little or no reactivity was observed in the lanes for recombinant HPV-16 L1 VLPs in Western blots with antisera to Sf-9S cell proteins or wild type baculovirus proteins, whereas polyclonal rabbit antisera to HPV-16 L1 proteins were positive for the recombinant HPV-16 L1 VLP lanes (FIG. 10A).

EXAMPLE 22

FORMULATION OF HPV-16 L1 VLP MONOVALENT VACCINE

[00227] Monovalent vaccines of HPV-16 VLPs were prepared by formulation of recombinant HPV-16 L1 VLPs manufactured by the methods in Example 18. Final bulk products of recombinant HPV-16 L1 VLPs were filtered through a 0.22 μm membrane aseptically and formulated at antigen concentrations of 20 and 100 mg/ml in phosphate-buffered saline alone or with alum adjuvant (Alhydrogel). The formulated products were dispensed as single dose units (0.5 cc) into sterile borosilicate glass vials (3 cc) silanized with dimethyldichlorosilane.

[00228] Also, final bulk products of recombinant HPV-16 L1 VLPs were formulated at antigen concentrations of 40 and 200 μg/ml in phosphate buffered saline, filtered, and dispensed

as single dose units (0.25 cc) into silanized glass vials (3 cc) for mixing just prior to immunization with an equal volume of the adjuvant MF-50 (Chiron). The final container products were labeled, checked for vial integrity, and stored at –20 or -70°C for final container vials containing VLP alone and 2-8°C for final container vials containing VLPs and alum adjuvant.

[00229] The final container products were subjected to safety and analytical testing as required by United States federal regulations and passed product specifications. Safety specifications included: (1) the absence of detectable microbial contaminants, spiroplasma, or mycoplasma, (2) endotoxins levels below 30 endotoxin units per ml, (3) the absence of adventitious agents by *in vitro* and *in vivo* testing, and (4) no adverse effects in adult mice and guinea pigs as part of the general safety tests. Analytical specifications included (1) the presence of HPV-16 L1 proteins with molecular weights between 50 and 60 kD at a purity $\geq$ 95% as determined by SDS-PAGE analysis coupled to scanning laser densitometry, (2) for identity testing, positive reactivity of proteins in the product to HPV-16 L1 antisera as determined by Western blot analysis, (3) for potency testing, positive reactivity of product at 100 µg/ml dilution with H16.V5 antisera for conformational epitopes as determined by ELISA testing, (4) for identity, purity, and potency testing, at least 75% of the product was present as intact VLPs as determined by analytical size exclusion chromatography and negative-stain electron microscopy, and for strength testing, protein content in the product at 20 and 100µg/ml with < 5% variance as determined by BCA assay.

[00230] Upon meeting product specifications, the final container vials were released for vaccination into healthy human volunteers to determine the safety and immunogenicity of the final container products as prophylactic vaccines for HPV-16 infection and disease.

EXAMPLE 23

CLINICAL INVESTIGATION OF HPV-16 VLP MONOVALENT VACCINE

[00231] The final container products as prepared by the methods of Example 22 were used to immunize healthy human volunteers in a double-blind, randomized clinical study at Johns Hopkins University to determine the safety and immunogenicity of the present invention as a monovalent vaccine to prevent HPV-16 infection and disease (Harro *et al.*, 2001). The study design encompassed two dosage regimens (10 and 50 μg VLP antigens) and four study arms including placebo, HPV-16 L1 VLPs alone, HPV-16 L1 VLPs + alum adjuvant, and HPV-16 L1 VLPs + MF-59 adjuvant. Female volunteers (72) who were sero-negative for HIV-1, had < four lifetime sexual partners, were not pregnant, and had normal cervical cytology and medical history, received three doses of vaccine or placebo intramuscularly in the deltoid muscle area at 0, 1, and 4 months. Serum samples were collected from vaccinees at one month post-immunization and evaluated by ELISA tests for the presence of HPV-16 antibodies. Vaccinees were followed up to one week post-immunization for presentation of adverse clinical signs.

[00232] The results of the clinical study indicated that the vaccine was well-tolerated as compared to placebo, as no major adverse effects were noted in any vaccinees. All vaccines receiving active vaccine seroconverted (4-fold antibody rise) for HPV-16. A dose-dependent immune response was observed in serum samples at 5 months post-immunization for those

vaccinees receiving VLPs alone or VLPs + Mf-59. However, no dose dependent response was seen in individuals receiving VLPs + alum. The antibody titers to HPV-16 neutralizing epitopes, as determined by ELISA tests of serum samples at five months post-immunization with 50 μg doses, were 1 x 10^4 E.U. for VLPs alone, 1 x 10^4 E.U. for VLPs + MF-59, and 2.2 x 10^3 E.U. for VLPs + alum. The neutralizing and ELISA antibody titers were shown to correlate well with 0.85 degree of confidence. Thus, the vaccines were shown to be well-tolerated and immunogenic. The antibody titers were approximately forty-fold higher than that associated with natural HPV-16 infections. ELISA antibody titers were demonstrated to be reliable correlates of HPV-16 neutralizing antibody titers. Lastly, HPV-16 L1 VLP vaccines consisting of VLPs alone at 50 μg per dose may provide protective immunity to prevent HPV-16 infection and resolve active HPV-16 infections and disease.

[00233] All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

What is claimed is:

1. A codon optimized polynucleotide encoding a viral capsid protein that self assembles into a virus-like particle that exhibits conformational antigenic epitopes capable of raising neutralizing antibodies, wherein the virus-like particle is expressed from a host cell extracellularly.

2. The codon optimized polynucleotide of Claim 1 comprising at least one of the following characteristics: (a) an increased number of nucleotide sequences that are utilized at high levels in insect cells, (b) a ratio of GC nucleotide pairs to AT nucleotide pairs of approximately 1:1, (c) a minimum number of palindromic and stem-loop DNA structures, and (d) a minimum number of transcription and post-transcription repressor elements.

3. The codon optimized polynucleotide of Claim 1 comprising a polynucleotide from an enveloped virus, or a non-enveloped virus.

4. The codon optimized polynucleotide of Claim 3, wherein the enveloped virus and the non-enveloped virus comprise rotavirus, calicivirus, hepatitis E virus, papillomavirus, influenza virus, hepatitis C virus, or retrovirus.

5. The codon optimized polynucleotide of Claim 4, wherein the papillomavirus is a human papillomavirus.

6. The codon optimized polynucleotide of Claim 5, wherein the human papillomavirus comprises genotypes HPV-16, HPV-18, HPV-45, HPV-31, HPV-33, HPV-35, HPV-51, HPV-52, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, or a combination thereof,

7. The codon optimized polynucleotide of Claim 5, wherein the human papillomavirus comprises SEQ ID No. 1, 6, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1.

8. A vector comprising the codon optimized polynucleotide of claim 1 operatively linked to an eukaryotic or a prokaryotic regulatory control element, capable of replication in prokaryotic host, eukaryotic host, or both.

9. The vector of Claim 8, wherein the vector is a baculovirus vector.

10. A host cell comprising the vector of Claim 8.

11. A pharmaceutical composition for treating, ameliorating, or preventing a papillomavirus related disease or disorder comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the virus-like particles are expressed from a host cell extracellularly, and an acceptable carrier or diluent.

12. The pharmaceutical composition of Claim 11, wherein the papillomavirus comprises a human papillomavirus.

13. The pharmaceutical composition of Claim 12, wherein the human papillomavirus comprises genotypes HPV-16, HPV-18, HPV-45, HPV-31, HPV-33, HPV-35, HPV-51, HPV-52, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, or a combination thereof,

14. The pharmaceutical composition of Claim 11, wherein the virus-like particles comprise HPV L1 VLPs.

15. A pharmaceutical composition comprising:

a) a polypeptide which is encoded by a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1;

b) a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1;

c) a vector carrying a polynucloetide molecule comprising SEQ ID No. 1, or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1;

d) a host cell genetically transformed with a polynucloetide molecule comprising SEQ ID No. 1, or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1; and

a pharmaceutically acceptable carrier or diluent.

16. A vaccine composition to induce immunity against a papillomavirus infection in humans comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the virus-like particles are expressed from a host cell extracellularly, and an adjuvant.

17. The vaccine composition of Claim 15, wherein the immunity is humoral immunity, cell mediated immunity, or both.

18. The vaccine composition of Claim 16, wherein the papillomavirus comprises human papillomavirus genotypes HPV-16, HPV-18, HPV-45, HPV-31, HPV-33, HPV-35, HPV-51, HPV-52, HPV-6, HPV-11, HPV-42, HPV-43, HPV-44, or a combination thereof,

19. The vaccine composition of Claim 16 comprising a monovalent or a multivalent formulation.

20. The vaccine composition of Claim 16, wherein the virus-like particles comprise HPV L1 VLPs.

21. A vaccine composition comprising:

a) a polypeptide which is encoded by a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1;

b) a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide having a sequence that is substantially homologous to SEQ ID No. 1;

c) a vector carrying a polynucleotide molecule comprising SEQ ID No. 1, or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1; or

d) a host cell genetically transformed with a polynucleotide molecule comprising SEQ ID No. 1,or a polynucleotide molecule having a sequence that is substantially homologous to SEQ ID No. 1; and

an adjuvant.

22. A diagnostic test kit for detection of papillomavirus infection comprising a multiplicity of virus-like particles that exhibit conformational antigenic epitopes, wherein the virus-like particles are expressed from a host cell extracellularly, and a detection agent comprising a detectable label.

23. The diagnostic test kit of Claim 22, wherein the papillomavirus infection is caused by one or more human papillomavirus genotypes.

24. The diagnostic test kit of Claim 22, wherein the virus-like particles comprise HPV L1 VLPs.

25. A method for preparing a codon optimized polynucleotide comprising one or more of the following steps:

(a) replacing codons that are underutilized in insect cells with codons that are utilized at high levels in insect cells, to create an initially-modified nucleotide sequence;

(b) modifying the initially-modified nucleotide sequence by choosing a preferred codon for the initially modified sequence, wherein:

(i) the ratio of GC nucleotide pairs to AT nucleotide pairs in the further-modified nucleotide sequence trends toward approximately 1:1;

(ii) the number of palindromic and stem-loop DNA structures in the further-modified nucleotide sequence is minimized; and

(iii) the number of transcription and post-transcription repressor elements are minimized.

26. The method of claim 25, wherein the polynucleotide comprises a human papillomavirus gene.

27. A method of treating, ameliorating, or preventing a papillomavirus related disease or disorder comprising, administering to an individual in need thereof an effective amount of the pharmaceutical composition of claim 11.

28. The method of Claim 27 wherein the papillomavirus infection is caused by one or more human papillomavirus genotypes.

29. A method of protecting an individual against a papillomavirus infection comprising, administering to the individual a prophylactically effective amount of the vaccine of Claim 16.

30. The method of Claim 29, wherein the papillomavirus infection is caused by one or more human papillomavirus genotypes.

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          5'         11         21         31         41         51         61         71
llgbseq      ATGTCTCTTT GGCTGCCTAG TGAGGCCACT GTCTACTTGC CTCCTGTCCC AGTATCTAAG GTTGTAAGCA CGGATGAATA
llnvax       ATGTCTCTTT GGCTGCCTAG TGAGGCCACT GTCTACTTGC CTCCTGTCCC AGTATCTAAG GTTGTAAGCA CGGATGAATA
lloptmzd     ATGTCCCTGT GGCTGCCCTG CGAGGCCACC GTCTACCTGC CCCCCGTCCC CGTGTCCAAG GTCGTCTCCA CCGATGAATA

          5'         91          1         11         21         31         41         51
llgbseq 81   TGTTGCACGC ACAAACATAT ATTATCATGC AGGAACATCC AGACTACTTG CAGTTGGACA TCCCTATTTT CCTATTAAAA
llnvax       TGTTGCACGC ACAAACATAT ATTATCATGC AGGAACATCC AGACTACTTG CAGTTGGACA TCCCTATTTT CCTATTAAAA
lloptmzd     CGTCGCTCGC ACCAACATCT ACTACCATGC TGGTACCTCC CGTCTCCTGG CTGTCGGTCA TCCCTACTTC CCCATCAAGA

          5'         71         81         91          1         11         21         31
llgbseq161   AACCTAACAA TAACAAAATA TTAGTTCCTA AAGTATCAGG ATTACAATAC AGGGTATTTA GAATACATTT ACCTGACCCC
llnvax       AACCTAACAA TAACAAAATA TTAGTTCCTA AAGTATCAGG ATTACAATAC AGGGTATTTA GAATACATTT ACCTGACCCC
lloptmzd     AGCCCAACAA CAACAAGATC CTCGTCCCCA AGGTCTCCGG TCTCCAAGTC CGTGTCTTCC GTATCCATCT CCCCGACCCC

          5'         51         61         71         81         91          1         11
llgbseq241   AATAAGTTTG GTTTTCCTGA CACCTCATTT TATAATCCAG ATACACAGCG GCTGGTTTGG GCCTGTGTAG GTGTTGAGGT
llnvax       AATAAGTTTG GTTTTCCTGA CACCTCATTT TATAATCCAG ATACACAGCG GCTGGTTTGG GCCTGTGTAG GTGTTGAGGT
lloptmzd     AACAAGTTCG GTTTCCCCGA CACCTCCTTC TACAACCCCG ATACCCAGCG CCTGTACTGG GCCTGCGTCG GTGTCGAGGT

          5'         31         41         51         61         71         81         91
llgbseq321   AGGTCGTGGT CAGCCATTAG GTGTGGGCAT TAGTGGCCAT CCTTTATTAA ATAAATTGGA TGACACAGAA AATGCTAGTG
llnvax       AGGTCGTGGT CAGCCATTAG GTGTGGGCAT TAGTGGCCAT CCTTTATTAA ATAAATTGGA TGACACAGAA AATGCTAGTG
lloptmzd     CGGTCGTGGT CAGCCCCTCG GTGTCGGCAT CTCCGGCCAC CCCCTCCTCA ACAAGCTCGA CGACACCGAG AACGCCTCCG

          5'         11         21         31         41         51         61         71
llgbseq401   CTTATGCAGC AAATGCAGGT GTGGATAATA GAGAATGTAT ATCTATGGAT TACAAACAAA CACAATTGTG TTTAATTGGT
llnvax       CTTATGCAGC AAATGCAGGT GTGGATAATA GAGAATGTAT ATCTATGGAT TACAAACAAA CACAATTGTG TTTAATTGGT
lloptmzd     CCTACGCCGC CAACGCCGGT GTCGACAACC GTGAGTGCAT GTCCATGGAC TACAAGCAGA CCCAGCTCTG CCTCATCGGT

          5'         91          1         11         21         31         41         51
llgbseq481   TGCAAACCAC CTATAGGGGA ACACTGGGGC AAAGGATCCC CATGTACCAA TGTTGCAGTA AATCCAGGTG ATTGTCCACC
llnvax       TGCAAACCAC CTATAGGGGA ACACTGGGGC AAAGGATCCC CATGTACCAA TGTTGCAGTA AATCCAGGTG ATTGTCCACC
lloptmzd     TGCAAGCCCC CCATCGGTGA GCACTGGGGT AAGGGTTCCC CCTGTACCAA CGTCGCCGTC AACCCCGGTG ACTGCCCCCC
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FIG. 1A

5' 71 81 91 1 11 21 31
11gbseq561 ATTAGAGTTA ATAAACACAG TTATTCAGGA TGGTGATATG GTTCATACTG GCTTTGGTGC TATGGACTTT ACTACATTAC
11nvax ATTAGAGTTA ATAAACACAG TTATTCAGGA TGGTGATATG GTTGATACTG GCTTTGGTGC TATGGACTTT ACTACATTAC
11optmzd CCTCGAGCTC ATCAACACCG TCATCCAGGA CGGTGACATG GTCGACACCG GCTTCGGTGC CATGGACTTC ACCACCCTCC

5' 51 61 71 81 91 1 11
11gbseq641 AGGCTAACAA AAGTGAAGTT CCACTGGATA TTTGTACATC TATTTGCAAA TATCCAGATT ATATTAAAAT GGTGTCAGAA
11nvax AGGCTAACAA AAGTGAAGTT CCACTGGATA TTTGTACATC TATTTGCAAA TATCCAGATT ATATTAAAAT GGTGTCAGAA
11optmzd AGGCCAACAA GTCCGAGGTC CCCCTCGACA TCTGCACCTC CATCTGCAAG TACCCCGACT ACATCAAGAT GGTCTCCGAG

5' 31 41 51 61 71 81 91
11gbseq721 CCATATGGCG ACAGCTTATT TTTTTATTTA CGAAGGGAAC AAATGTTTGT TAGACATTTA TTTAATAGGG CTGGTACTGT
11nvax CCATATGGCG ACAGCTTATT TTTTTATTTA CGAAGGGAAC AAATGTTTGT TAGACATTTA TTTAATAGGG CTGGTGCTGT
11optmzd CCCTACGGTG ACTCCCTCTT CTTCTACCTC CGCCGCGAGC AGATGTTCGT CCGCCACCTC TTCAACCGCG CCGGTGCTGT

5' 11 21 31 41 51 61 71
11gbseq801 TGGTGAAAAT GTACCAGACG ATTTATACAT TAAAGGCTCT GGGTCTACTG CAAATTTAGC CAGTTCAAAT TATTTTCCTA
11nvax TGGTGAAAAT GTACCAGACG ATTTATACAT TAAAGGCTCT GGGTCTACTG CAAATTTAGC CAGTTCAAAT TATTTTCCTA
11optmzd CGGTGAGAAC GTCCCCGACG ACCTCTACAT CAAGGGTTCC GGTTCCACCG CCAACCTCGC TTCCTCCAAC TACTTCCCCA

5' 91 1 11 21 31 41 51
11gbseq881 CACCTAGTGG TTCTATGGTT ACCTCTGATG CCCAAATATT CAATAAACCT TATTGGTTAC AACGAGCACA GGGCCACAAT
11nvax CACCTAGTGG TTCTATGGTT ACCTCTGATG CCCAAATATT CAATAAACCT TATTGGTTAC AACGAGCACA GGGCCACAAT
11optmzd CCCCCTCCGG TTCCATGGTC ACCTCCGACG CCCAGATCTT CAACAAGCCC TACTGGCTCC AGCGCGCTCA GGGTCACAAC

5' 71 81 91 1 11 21 31
11gbseq961 AATGGCATTT GTTGGGGTAA CCAACTATTT GTTACTGTTG TTGATACTAC ACGCAGTACA AATATGTCAT TATGTGCTGC
11nvax AATGGCATTT GTTGGGGTAA CCAACTATTT GTTACTGTTG TTGATACTAC ACGCAGTACA AATATGTCAT TATGTGCTGC
11optmzd AACGGTATCT GCTGGGGTAA CCAGCTCTTC GTCACCGTCG TCGACACCAC CCGCTCCACC AACATGTCCC TCTGCGCCGC

5' 51 61 71 81 91 1 11
11gbseq1041 CATATCTACT TCAGAAACTA CATATAAAAA TACTAACTTT AAGGAGTACC TACGACATGG GGAGGAATAT GATTTACAGT
11nvax CATATCTACT TCAGAAACTA CATATAAAAA TACTAACTTT AAGGAGTACC TACGACATGG GGAGGAATAT GATTTACAGT
11optmzd CATCTCCACC TCCGAGACCA CCTACAAGAA CACCAACTTC AAGGAGTACC TCCGCCACGG TGAGGAGTAC GACCTCCAGT

FIG. 1B

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           5'         31         41         51         61         71         81         91
llgbseq121 TTATTTTTCA ACTGTGCAAA ATAACCTTAA CTGCAGACGT TATGACATAC ATACATTCTA TGAATTCCAC TATTTTGGAG
llnvax     TTATTTTTCA ACTGTGCAAA ATAACCTTAA CTGCAGACGT TATGACATAC ATACATTCTA TGAATTCCAC TATTTTGGAG
lloptmzd   TCATCTTCCA GCTCTGCAAG ATCACCCTCA CCGCCGACGT CATGACCTAC ATCCACTCCA TGAACTCCAC CATCCTCGAG

           5'         11         21         31         41         51         61         71
llgbseq201 GACTGGAATT TTGGTCTACA ACCTCCCCCA GGAGGCACAC TAGAAGATAC TTATAGGTTT GTAACCCAGG CAATTGCTTG
llnvax     GACTGGAATT TTGGTCTACA ACCTCCCCCA GGAGGCACAC TAGAAGATAC TTATAGGTTT GTAACATCCC AGGCAATTGC
lloptmzd   GACTGGAACT TCGGTCTCCA GCCCCCCCCG GGTGGTACCC TCGAGGACAC CTACCGCTTC GTCACCTCCC AGGCCATCGC

           5'         91         1          11         21         31         41         51
llgbseq281 TCAAAAACAT ACACCTCCAG CACCTAAAGA AGATGATCCC CTTAAAAAAT ACACTTTTTG GGAAGTAAAT TTAAAGGAAA
llnvax     TTGTCAAAAA CATACACCTC CAGCACCTAA AGAAGATCCC CTTAAAAAAT ACACTTTTTG GGAAGTAAAT TTAAAGGAAA
lloptmzd   CTGCCAGAAG CACACCCCCC CCGCCCCCAA GGAGGACCCC CTCAAGAAGT ACACCTTCTG GGAGGTCAAC CTCAAGGAGA

           5'         71         81         91         1          11         21         31
llgbseq361 AGTTTTCTGC AGACCTAGAT CAGTTTCCTT TAGGACGCAA ATTTTTACTA CAAGCAGGAT TGAAGGCCAA ACCAAAATTT
llnvax     AGTTTTCTGC AGACCTAGAT CAGTTTCCTT TAGGACGCAA ATTTTTACTA CAAGCAGGAT TGAAGGCCAA ACCAAAATTT
lloptmzd   AGTTCTCCGC CGACCTCGAC CAGTTCCCCC TCGGTCGCAA GTTCCTCCTC CAGGCCGGTC TCAAGGCCAA GCCCAAGTTC

           5'         51         61         71         81         91         1          11
llgbseq441 ACATTAGGAA AACGAAAACG TACACCCACC ACCTCATCTA CCTCTACAAC TGCTAAACGC AAAAAACGTA AGCTGTAA--
llnvax     ACATTAGGAA AACGAAAAGC TACACCCACC ACCTCATCTA CCTCTACAAC TGCTAAACGC AAAAAACGTA AGCTGTAA--
lloptmzd   ACCCTCGGTA AGCGCAAGGC CACCCCCACC ACCTCCTCCA CCTCCACCAC CGCCAAGCGC AAGAAGCGCA AGCTC-----

           5'         31         41         51         61         71         81         91
llgbseq521 ---------- ---------- ----------
llnvax     ---------- ---------- ----------
lloptmzd   ---------- ---------- ----------
```

FIG. 1C

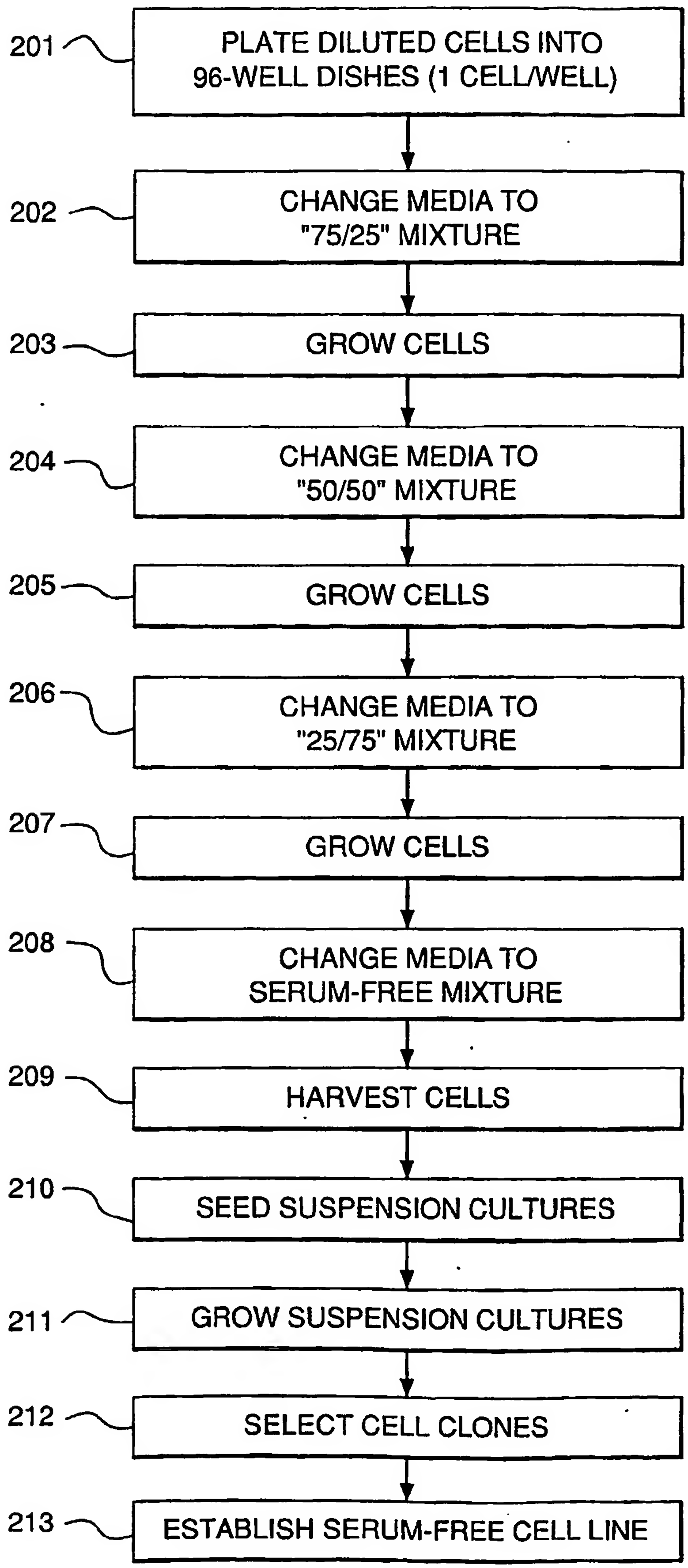

201
PLATE DILUTED CELLS INTO 96-WELL DISHES (1 CELL/WELL)
202
CHANGE MEDIA TO "75/25" MIXTURE
203
GROW CELLS
204
CHANGE MEDIA TO "50/50" MIXTURE
205
GROW CELLS
206
CHANGE MEDIA TO "25/75" MIXTURE
207
GROW CELLS
208
CHANGE MEDIA TO SERUM-FREE MIXTURE
209
HARVEST CELLS
210
SEED SUSPENSION CULTURES
211
GROW SUSPENSION CULTURES
212
SELECT CELL CLONES
213
ESTABLISH SERUM-FREE CELL LINE

FIG. 2

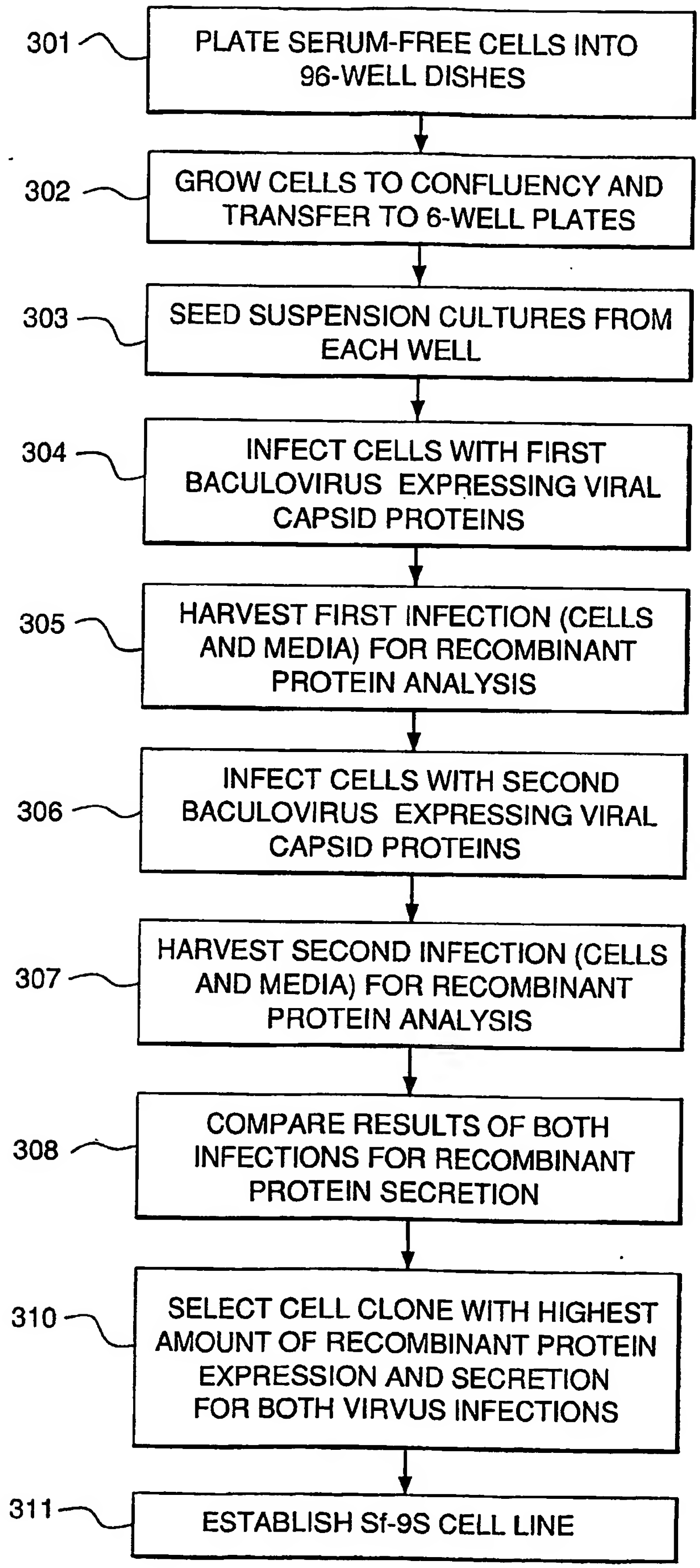

301
PLATE SERUM-FREE CELLS INTO 96-WELL DISHES
302
GROW CELLS TO CONFLUENCY AND TRANSFER TO 6-WELL PLATES
303
SEED SUSPENSION CULTURES FROM EACH WELL
304
INFECT CELLS WITH FIRST BACULOVIRUS EXPRESSING VIRAL CAPSID PROTEINS
305
HARVEST FIRST INFECTION (CELLS AND MEDIA) FOR RECOMBINANT PROTEIN ANALYSIS
306
INFECT CELLS WITH SECOND BACULOVIRUS EXPRESSING VIRAL CAPSID PROTEINS
307
HARVEST SECOND INFECTION (CELLS AND MEDIA) FOR RECOMBINANT PROTEIN ANALYSIS
308
COMPARE RESULTS OF BOTH INFECTIONS FOR RECOMBINANT PROTEIN SECRETION
310
SELECT CELL CLONE WITH HIGHEST AMOUNT OF RECOMBINANT PROTEIN EXPRESSION AND SECRETION FOR BOTH VIRVUS INFECTIONS
311
ESTABLISH Sf-9S CELL LINE

FIG. 3

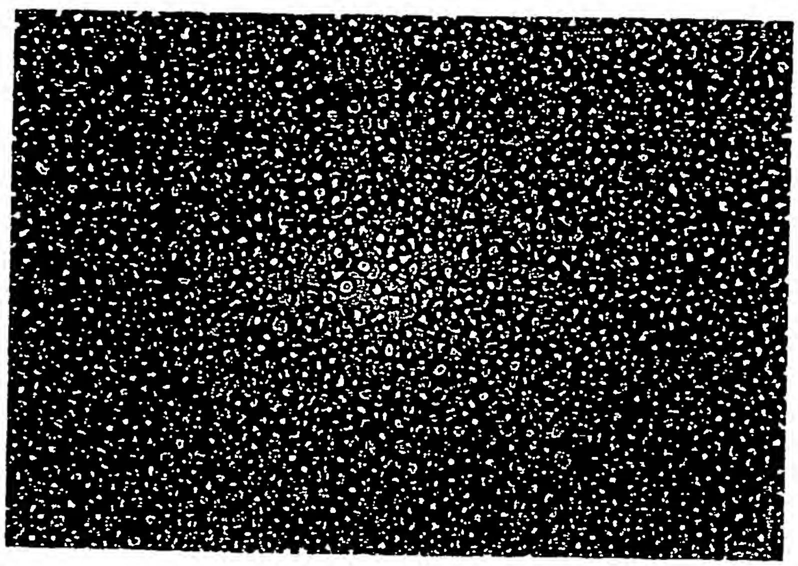


FIG. 4

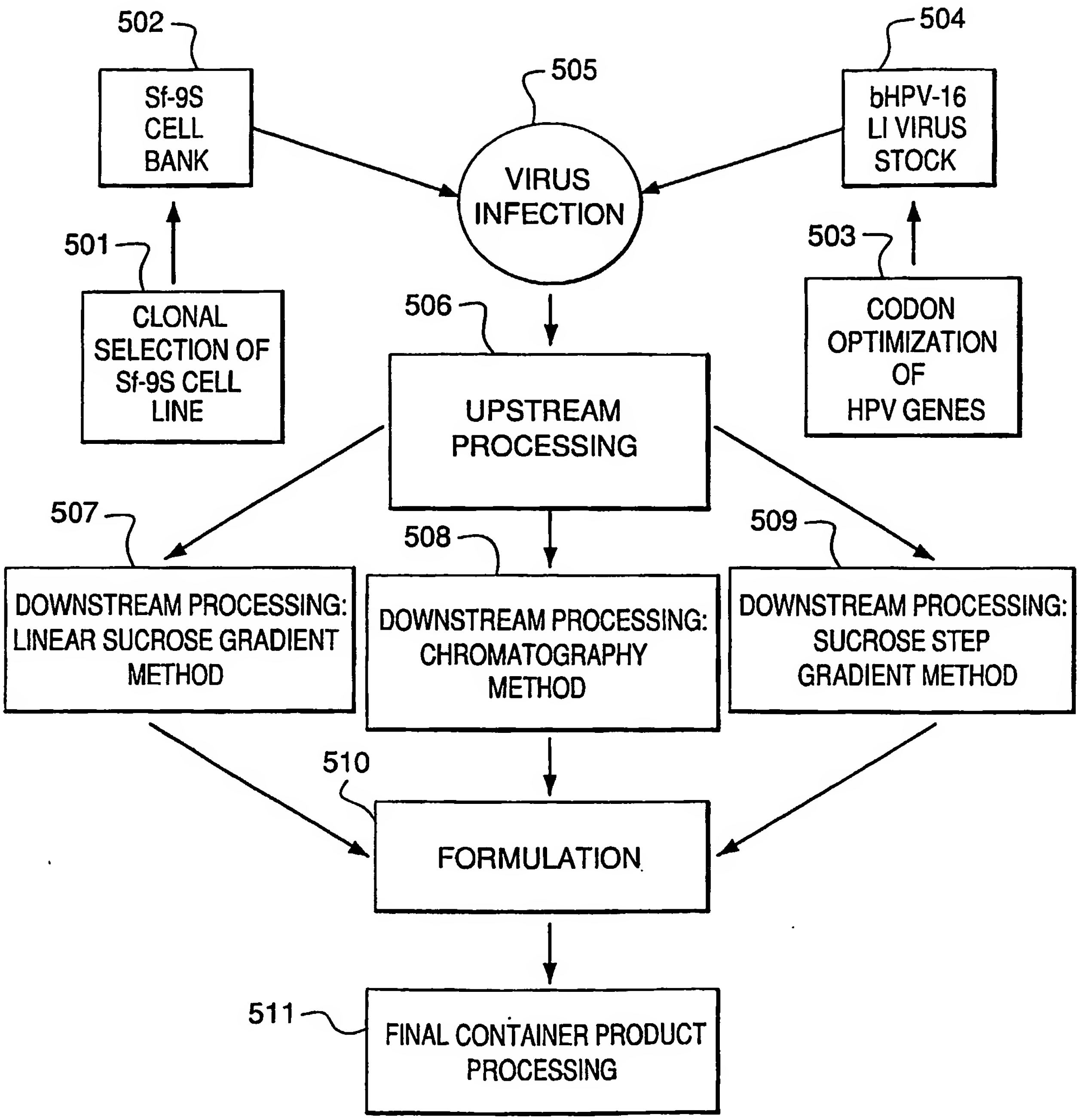
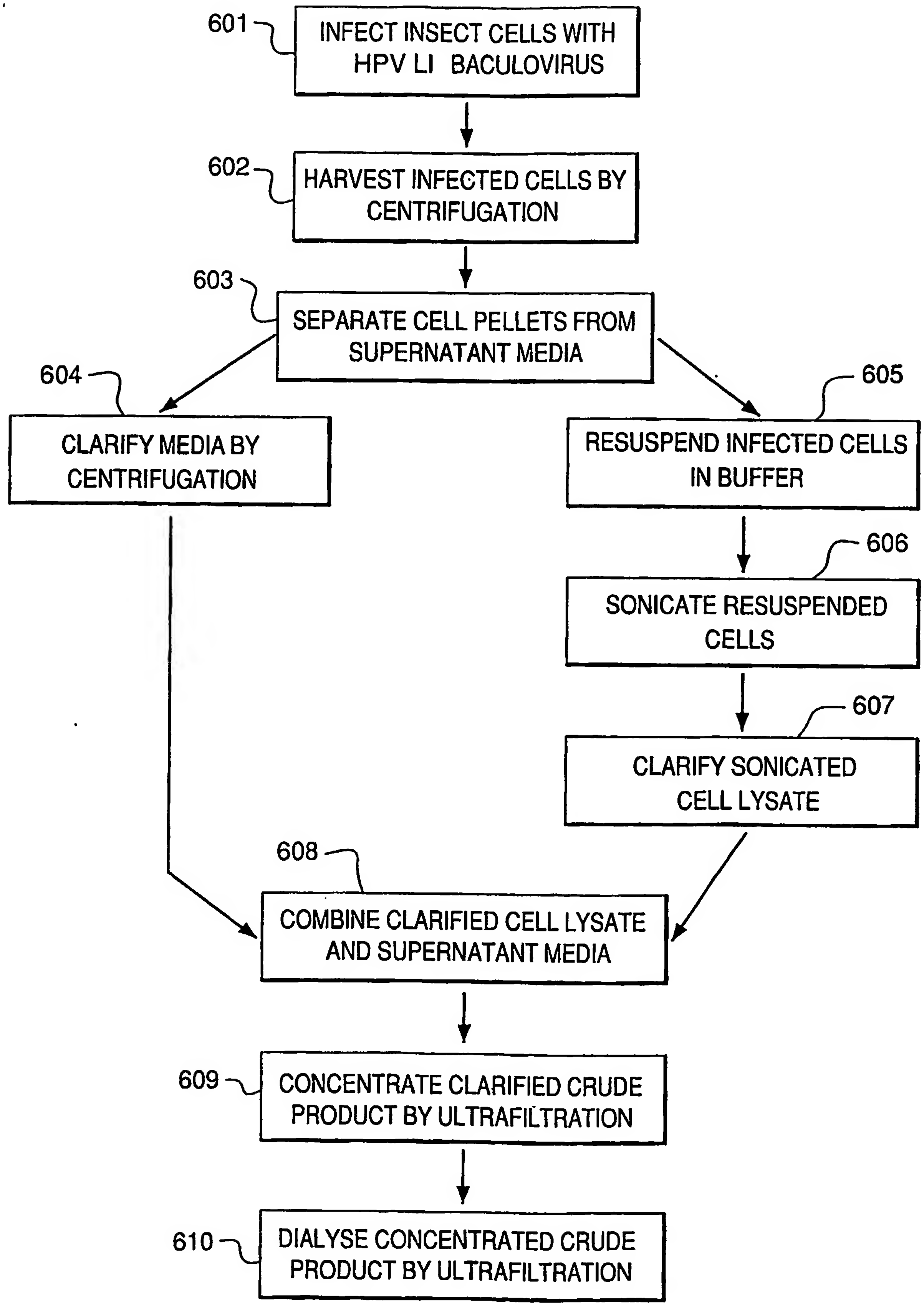

502
Sf-9S CELL BANK
505
VIRUS INFECTION
504
bHPV-16 LI VIRUS STOCK
501
CLONAL SELECTION OF Sf-9S CELL LINE
506
UPSTREAM PROCESSING
503
CODON OPTIMIZATION OF HPV GENES
507
DOWNSTREAM PROCESSING: LINEAR SUCROSE GRADIENT METHOD
508
DOWNSTREAM PROCESSING: CHROMATOGRAPHY METHOD
509
DOWNSTREAM PROCESSING: SUCROSE STEP GRADIENT METHOD
510
FORMULATION
511
FINAL CONTAINER PRODUCT PROCESSING

FIG. 5



601
INFECT INSECT CELLS WITH HPV LI BACULOVIRUS
602
HARVEST INFECTED CELLS BY CENTRIFUGATION
603
SEPARATE CELL PELLETS FROM SUPERNATANT MEDIA
604
CLARIFY MEDIA BY CENTRIFUGATION
605
RESUSPEND INFECTED CELLS IN BUFFER
606
SONICATE RESUSPENDED CELLS
607
CLARIFY SONICATED CELL LYSATE
608
COMBINE CLARIFIED CELL LYSATE AND SUPERNATANT MEDIA
609
CONCENTRATE CLARIFIED CRUDE PRODUCT BY ULTRAFILTRATION
610
DIALYSE CONCENTRATED CRUDE PRODUCT BY ULTRAFILTRATION

FIG 6

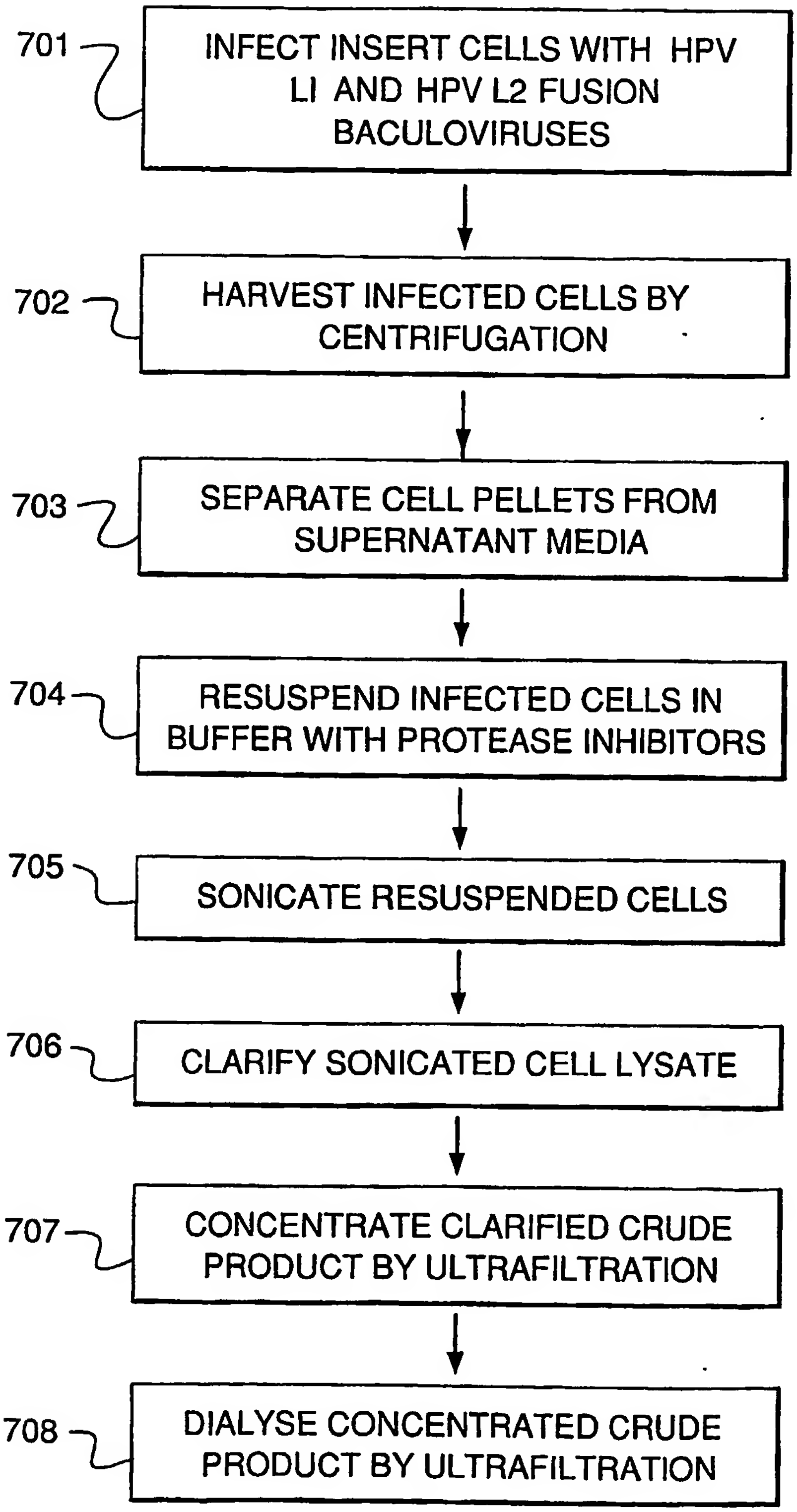

701
INFECT INSERT CELLS WITH HPV LI AND HPV L2 FUSION BACULOVIRUSES
702
HARVEST INFECTED CELLS BY CENTRIFUGATION
703
SEPARATE CELL PELLETS FROM SUPERNATANT MEDIA
704
RESUSPEND INFECTED CELLS IN BUFFER WITH PROTEASE INHIBITORS
705
SONICATE RESUSPENDED CELLS
706
CLARIFY SONICATED CELL LYSATE
707
CONCENTRATE CLARIFIED CRUDE PRODUCT BY ULTRAFILTRATION
708
DIALYSE CONCENTRATED CRUDE PRODUCT BY ULTRAFILTRATION

FIG. 7

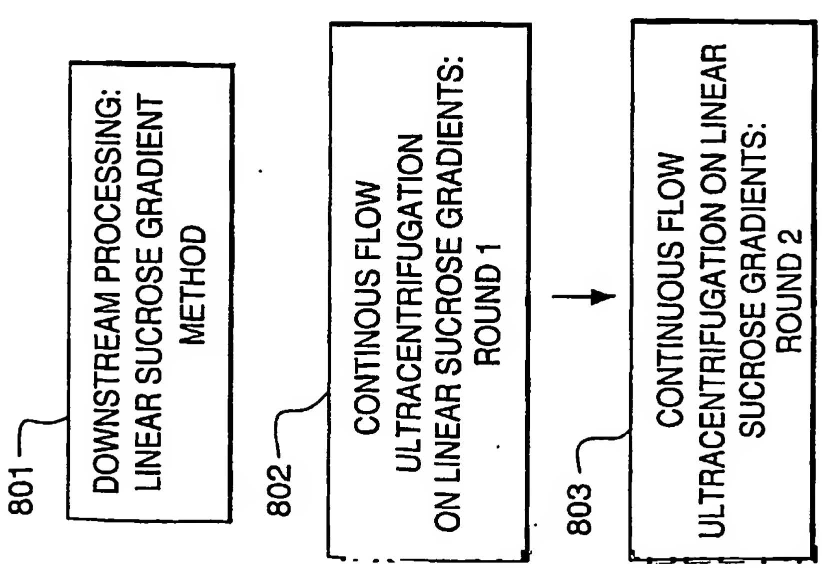


FIG. 8A

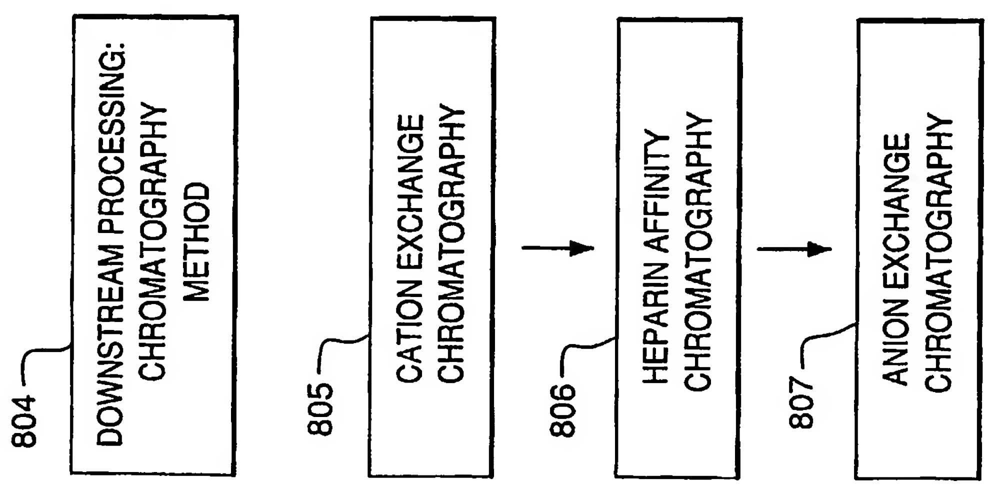


FIG. 8B

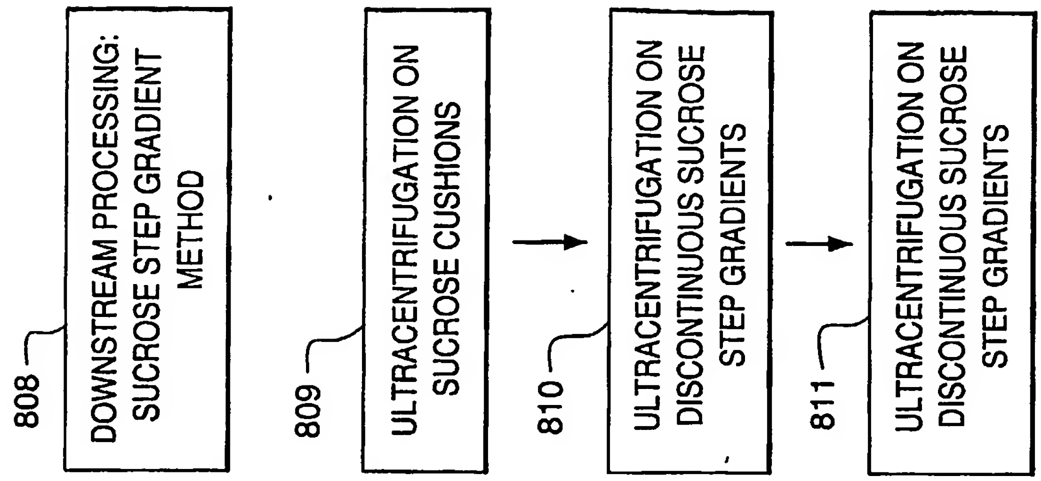


FIG. 8C

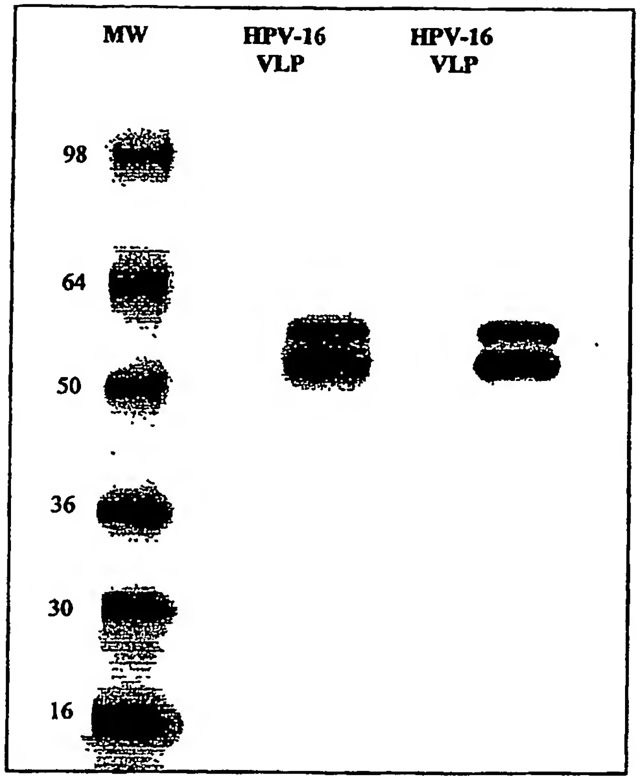


FIG. 9

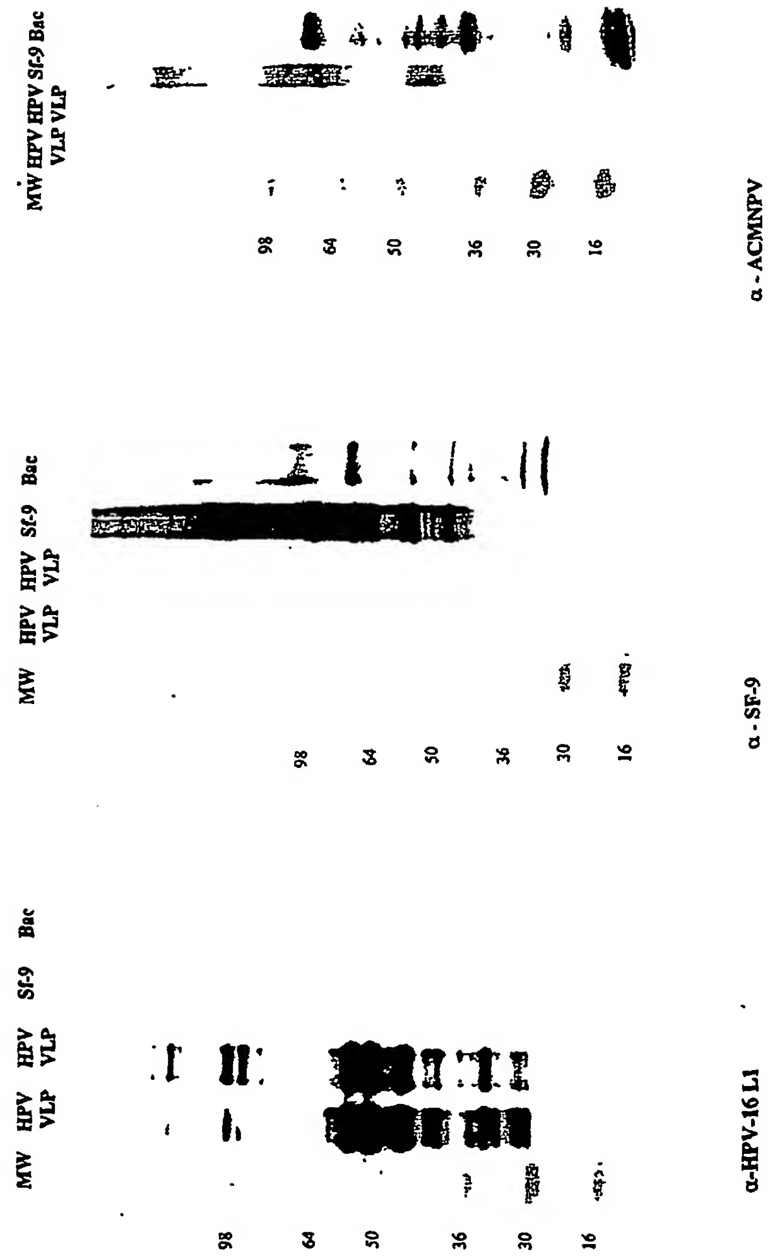

MW HPV HPV Sf-9 Bac
VLP VLP
98
64
50
36
30
16
α - ACMNPV
MW HPV HPV Sf-9 Bac
VLP VLP
98
64
50
36
30
16
α - SF-9
MW HPV HPV Sf-9 Bac
VLP VLP
98
64
50
36
30
16
α-HPV-16 L1

FIG. 10

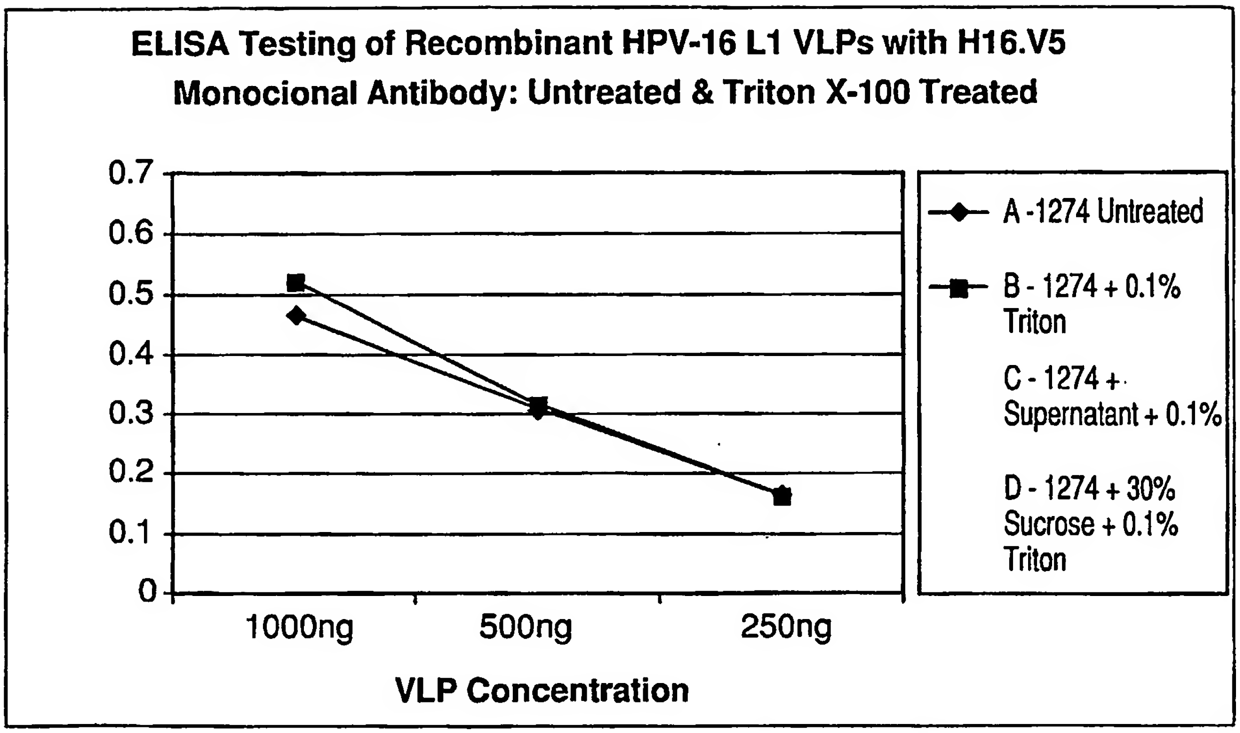

ELISA Testing of Recombinant HPV-16 L1 VLPs with H16.V5 Monoclonal Antibody: Untreated & Triton X-100 Treated
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1000ng
500ng
250ng
VLP Concentration
A -1274 Untreated
B - 1274 + 0.1% Triton
C - 1274 + Supernatant + 0.1%
D - 1274 + 30% Sucrose + 0.1% Triton

FIG. 11

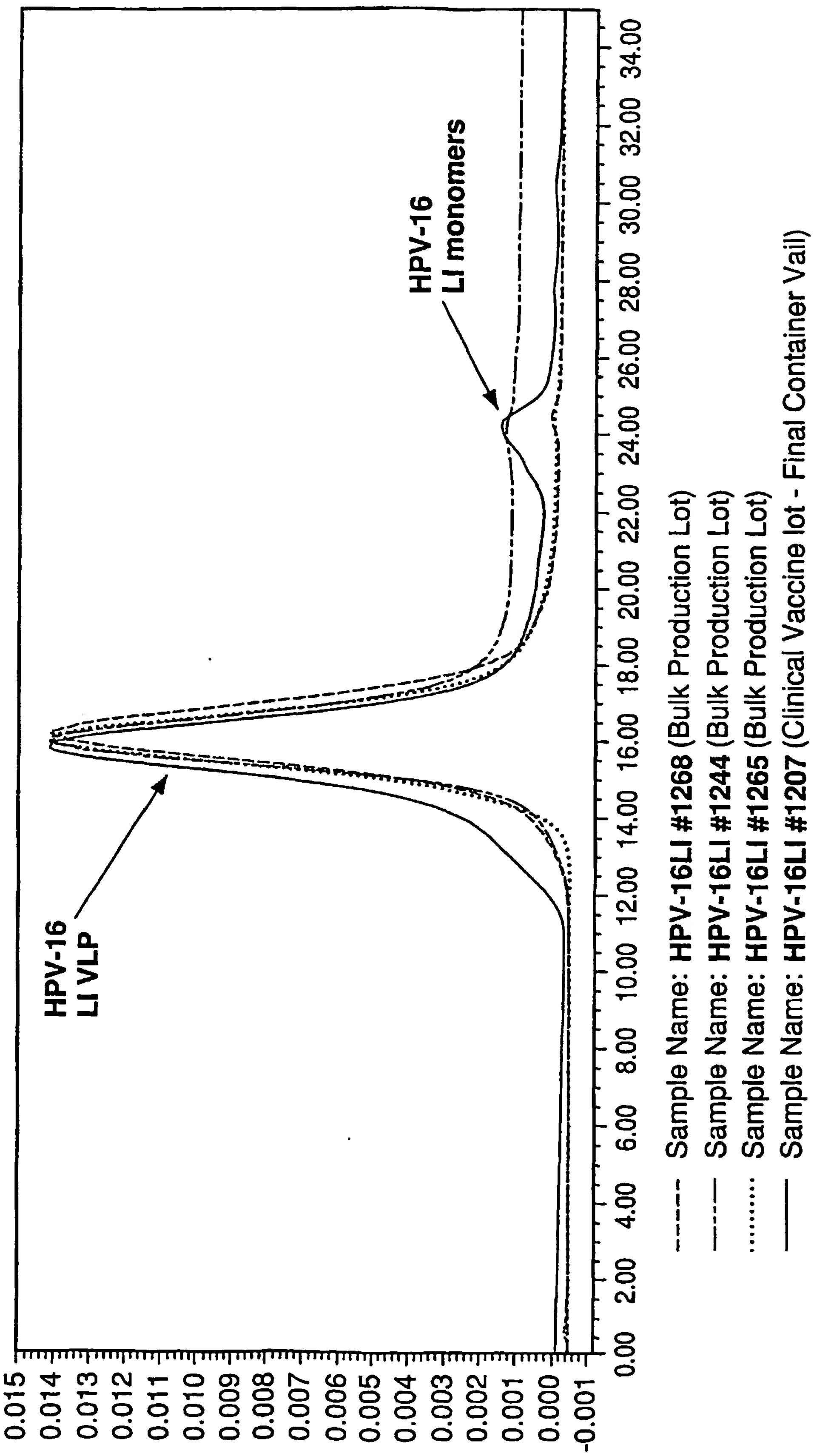

HPV-16
LI VLP
HPV-16
LI monomers
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
-0.001
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
Sample Name: **HPV-16LI #1268** (Bulk Production Lot)
Sample Name: **HPV-16LI #1244** (Bulk Production Lot)
Sample Name: **HPV-16LI #1265** (Bulk Production Lot)
Sample Name: **HPV-16LI #1207** (Clinical Vaccine lot - Final Container Vail)

FIG. 12

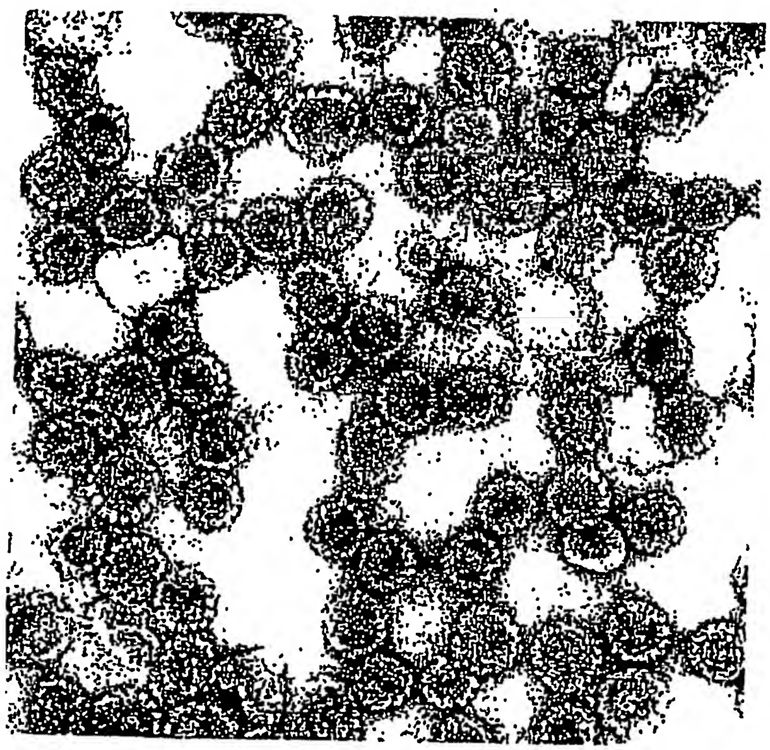


FIG. 13

SEQUENCE LISTING

<110> Robin A. Robinson

<120> Optimization of Gene Sequences of
Virus-Like Particles for Expression in Insect Cells

<130> 44149-2PC1

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<151> 2002-02-14

<150> US 60/356,161
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<150> US 60/356,118
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<150> US 60/356,123
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<151> 2002-02-14

<150> US 60/356,150
<151> 2002-02-14

<150> US 60/356,151
<151> 2002-02-14

<150> US 60/356,152
<151> 2002-02-14

<160> 13

<170> FastSEQ for Windows Version 4.0

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<211> 1515
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L1 codon optimized sequence

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cgtctcctgg ctgtcggtca tccctacttc cccatcaaga agcccaacaa caacaagatc 180
ctcgtcccca aggtctccgg tctccaagtc cgtgtcttcc gtatccatct ccccgacccc 240
aacaagttcg gtttccccga cacctccttc tacaaccccg atacccagcg cctgtactgg 300
gcctgcgtcg gtgtcgaggt cggtcgtggt cagcccctcg gtgtcggcat ctccggccac 360
cccctcctca acaagctcga cgacaccgag aacgcctccg cctacgccgc caacgccggt 420
gtcgacaacc gtgagtgcat ctccatggac tacaagcaga cccagctctg cctcatcggt 480
tgcaagcccc ccatcggtga gcactggggt aagggttccc cctgcaccaa cgtcgccgtc 540
aaccccggtg actgccccc cctcgagctc atcaacaccg tcatccagga cggtgacatg 600
gtcgacaccg gtttcggtgc catggacttc accaccctcc aggccaacaa gtccgaggtc 660
cccctcgaca tctgcacctc catctgcaag taccccgact acatcaagat ggtctccgag 720
ccctacggtg actccctctt cttctacctc cgccgcgagc agatgttcgt ccgccacctc 780
ttcaaccgcg ccggtgctgt cggtgagaac gtccccgacg acctctacat caagggttcc 840
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aacatgtccc tctgcgccgc catctccacc tccgagacca cctacaagaa caccaacttc 1080
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<211> 1413
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L2 codon optimized sequence

<400> 2
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acctgcaagc aggccggtac ctgccccccc gacatcatcc ccaaggtcga gggtaagacc 120
atcgccgacc agatcctcca gtacggttcc atgggtgtct tcttcggtgg tctcggtatc 180
ggtaccggtt ccggtaccgg tggtcgtacc ggttacatcc ccctcggtac ccgtcccccc 240
accgccaccg acaccctcgc ccccgtccgt ccccccctca ccgtcgaccc cgtcggtccc 300
tccgacccct ccatcgtctc cctcgtcgag gagacctcct tcatcgacgc cggtgccccc 360
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cccaccttca ccgacccctc cgtcctccag ccccccaccc ccgccgagac cggtggtcac 540
ttcaccctct cctcctccac catctccacc cacaactacg aggagatccc catggacacc 600
tttatcgtct ccaccaaccc caacaccgtc acctcctcca cccccatccc cggttcccgt 660
cccgtcgccc gtctgggcct ctactcccgt accacccagc aggtcaaggt cgtcgacccc 720
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gacgtcgaca acaccctcta cttctcctcc aacgacaact ccatcaacat cgcccccgac 840
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atccgctact cccgtatcgg taacaagcag accctccgta cccgttccgg taagtccatc 960
ggtgccaagg tccactacta ctacgacttc tccaccatcg accccgccga ggagatcgag 1020
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cgtctcccct acttcttctc cgacgtctcc tga 1413

<210> 3
<211> 1466
<212> DNA
<213> Artificial Sequence

<220>

<223> HPV-16 L2/E7 fusion gene codon optimized sequence

<400> 3
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aaacatgcaa acaggcaggt acatgtccac ctgacattat acctaaggtt gaaggcaaaa 120
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aatatatgtt agatttgcaa ccagag 1466

<210> 4
<211> 2805
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L2/E7/E2 fusion gene codon optimized sequence

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ccttttggtg gtgcatacaa tattccttta gtatcaggtc ctgatatacc cattaatata 1260
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agctcagagg aggaggatga aatagatggt ccagctggac aagcagaacc ggacagagcc 1560
cattacaata ttgtaacctt ttgttgcaag tgtgactcta cgcttcggtt gtgcgtacaa 1620
agcacacacg tagacattcg tactttggaa gacctgttaa tgggcacact aggaattgtg 1680
tgccccatct gttctcagaa accatcgatg gagactcttt gccaacgttt aaatgtgtgt 1740

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tattggaaac acatgcgcct agaatgtgct atttattaca aggccagaga aatgggattt 1860
aaacatatta accaccaggt ggtgccaaca ctggctgtat caaagaataa agcattacaa 1920
gcaattgaac tgcaactaac gttagaaaca atatataact cacaatatag taatgaaaag 1980
tggacattac aagacgttag ccttgaagtg tatttaactg caccaacagg atgtataaaa 2040
aaacatggat atacagtgga agtgcagttt gatggagaca tatgcaatac aatgcattat 2100
acaaactgga cacatatata tatttgtgaa gaagcatcag taactgtggt agagggtcaa 2160
gttgactatt atggtttata ttatgttcat gaaggaatac gaacatattt tgtgcagttt 2220
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gtaatattat gtcctacatc tgtgtttagc agcaacgaag tatcctctcc tgaaattatt 2340
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gaagaaacac agacgactat ccagcgacca agatcagagc cagacaccgg aaacccctgc 2460
cacaccacta agttgttgca cagagactca gtggacagtg ctccaatcct cactgcattt 2520
aacagctcac acaaaggacg gattaactgt aatagtaaca ctacacccat agtacattta 2580
aaagtggatg ctaatacttt aaaatgttta agatatagat ttaaaaagca ttgtacattg 2640
tatactgcag tgtcgtctac atggcattgg acaggacata atgtaaaaca taaaagtgca 2700
attgttacac ttacatatga tagtgaatgg caacgtgacc aatttttgtc tcaagttaaa 2760
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<210> 5
<211> 1611
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L2/E6 fusion gene codon optimized sequence

<400> 5

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accaccccog ccatcctcga catcaacaac accgtcacca ccgtcaccac ccacaacaac 480
cccaccttca ccgacccctc cgtcctccag cccccaccc ccgccgagac cggtggtcac 540
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gccttcgtca ccacccccac caagctcatc acctacgaca accccgccta cgagggtatc 780
gacgtcgaca acaccctcta cttctcctcc aacgacaact ccatcaacat cgcccccgac 840
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ggtgccaagg tccactacta ctacgacttc tccaccatcg accccgccga ggagatcgag 1020
ctccagacca tcacccccctc cacctacacc accacctccc acgccgcctc cccccacctcc 1080
atcaacaacg gtctctacga catctacgcc gacgacttca tcaccgacac ctccaccacc 1140
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cccttcggtg gcgcctacaa catccccctc gtctccggtc ccgacatccc catcaacatc 1260
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gacccccagg agcgtccccg taagctcccc cagctctgca ccgagctcca gaccaccatc 1500
cacgacatca tcctcgagtg cgtctactgc aagcagcagc tcctgcgtcg tgaggtctac 1560
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<210> 6
<211> 505
<212> PRT
<213> Artificial Sequence

<220>
<223> HPV-16 L1 protein

<400> 6

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Tyr Phe Pro Ile Lys Lys Pro Asn Asn Asn Lys Ile Leu Val Pro Lys
50 55 60
Val Ser Gly Leu Gln Tyr Arg Val Phe Arg Ile His Leu Pro Asp Pro
65 70 75 80
Asn Lys Phe Gly Phe Pro Asp Thr Ser Phe Tyr Asn Pro Asp Thr Gln
85 90 95
Arg Leu Val Trp Ala Cys Val Gly Val Glu Val Gly Arg Gly Gln Pro
100 105 110
Leu Gly Val Gly Ile Ser Gly His Pro Leu Leu Asn Lys Leu Asp Asp
115 120 125
Thr Glu Asn Ala Ser Ala Tyr Ala Ala Asn Ala Gly Val Asp Asn Arg
130 135 140
Glu Cys Ile Ser Met Asp Tyr Lys Gln Thr Gln Leu Cys Leu Ile Gly
145 150 155 160
Cys Lys Pro Pro Ile Gly Glu His Trp Gly Lys Gly Ser Pro Cys Thr
165 170 175
Asn Val Ala Val Asn Pro Gly Asp Cys Pro Pro Leu Glu Leu Ile Asn
180 185 190
Thr Val Ile Gln Asp Gly Asp Met Val Asp Thr Gly Phe Gly Ala Met
195 200 205
Asp Phe Thr Thr Leu Gln Ala Asn Lys Ser Glu Val Pro Leu Asp Ile
210 215 220
Cys Thr Ser Ile Cys Lys Tyr Pro Asp Tyr Ile Lys Met Val Ser Glu
225 230 235 240
Pro Tyr Gly Asp Ser Leu Phe Phe Tyr Leu Arg Arg Glu Gln Met Phe
245 250 255
Val Arg His Leu Phe Asn Arg Ala Gly Ala Val Gly Glu Asn Val Pro
260 265 270
Asp Asp Leu Tyr Ile Lys Gly Ser Gly Ser Thr Ala Asn Leu Ala Ser
275 280 285
Ser Asn Tyr Phe Pro Thr Pro Ser Gly Ser Met Val Thr Ser Asp Ala
290 295 300
Gln Ile Phe Asn Lys Pro Tyr Trp Leu Gln Arg Ala Gln Gly His Asn
305 310 315 320
Asn Gly Ile Cys Trp Gly Asn Gln Leu Phe Val Thr Val Val Asp Thr
325 330 335
Thr Arg Ser Thr Asn Met Ser Leu Cys Ala Ala Ile Ser Thr Ser Glu
340 345 350
Thr Thr Tyr Lys Asn Thr Asn Phe Lys Glu Tyr Leu Arg His Gly Glu
355 360 365
Glu Tyr Asp Leu Gln Phe Ile Phe Gln Leu Cys Lys Ile Thr Leu Thr
370 375 380
Ala Asp Val Met Thr Tyr Ile His Ser Met Asn Ser Thr Ile Leu Glu
385 390 395 400
Asp Trp Asn Phe Gly Leu Gln Pro Pro Pro Gly Gly Thr Leu Glu Asp
405 410 415
Thr Tyr Arg Phe Val Thr Ser Gln Ala Ile Ala Cys Gln Lys His Thr
420 425 430
Pro Pro Ala Pro Lys Glu Asp Pro Leu Lys Lys Tyr Thr Phe Trp Glu
435 440 445
Val Asn Leu Lys Glu Lys Phe Ser Ala Asp Leu Asp Gln Phe Pro Leu
450 455 460
Gly Arg Lys Phe Leu Leu Gln Ala Gly Leu Lys Ala Lys Pro Lys Phe
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Thr Leu Gly Lys Arg Lys Ala Thr Pro Thr Thr Ser Ser Thr Ser Thr
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Thr Ala Lys Arg Lys Lys Arg Lys Leu
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<211> 470
<212> PRT
<213> Artificial Sequence

<220>
<223> HPV-16 L2 protein

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Ile Pro Lys Val Glu Gly Lys Thr Ile Ala Asp Gln Ile Leu Gln Tyr
35 40 45
Gly Ser Met Gly Val Phe Phe Gly Gly Leu Gly Ile Gly Thr Gly Ser
50 55 60
Gly Thr Gly Gly Arg Thr Gly Tyr Ile Pro Leu Gly Thr Arg Pro Pro
65 70 75 80
Thr Ala Thr Asp Thr Leu Ala Pro Val Arg Pro Pro Leu Thr Val Asp
85 90 95
Pro Val Gly Pro Ser Asp Pro Ser Ile Val Ser Leu Val Glu Glu Thr
100 105 110
Ser Phe Ile Asp Ala Gly Ala Pro Thr Ser Val Pro Ser Ile Pro Pro
115 120 125
Asp Val Ser Gly Phe Ser Ile Thr Thr Ser Thr Asp Thr Thr Pro Ala
130 135 140
Ile Leu Asp Ile Asn Asn Thr Val Thr Thr Val Thr Thr His Asn Asn
145 150 155 160
Pro Thr Phe Thr Asp Pro Ser Val Leu Gln Pro Pro Thr Pro Ala Glu
165 170 175
Thr Gly Gly His Phe Thr Leu Ser Ser Ser Thr Ile Ser Thr His Asn
180 185 190
Tyr Glu Glu Ile Pro Met Asp Thr Phe Ile Val Ser Thr Asn Pro Asn
195 200 205
Thr Val Thr Ser Ser Thr Pro Ile Pro Gly Ser Arg Pro Val Ala Arg
210 215 220
Leu Gly Leu Tyr Ser Arg Thr Thr Gln Gln Val Lys Val Val Asp Pro
225 230 235 240
Ala Phe Val Thr Thr Pro Thr Lys Leu Ile Thr Tyr Asp Asn Pro Ala
245 250 255
Tyr Glu Gly Ile Asp Val Asp Asn Thr Leu Tyr Phe Ser Ser Asn Asp
260 265 270
Asn Ser Ile Asn Ile Ala Pro Asp Pro Asp Phe Leu Asp Ile Val Ala
275 280 285
Leu His Arg Pro Ala Leu Thr Ser Arg Arg Thr Gly Ile Arg Tyr Ser
290 295 300
Arg Ile Gly Asn Lys Gln Thr Leu Arg Thr Arg Ser Gly Lys Ser Ile
305 310 315 320
Gly Ala Lys Val His Tyr Tyr Tyr Asp Phe Ser Thr Ile Asp Pro Ala
325 330 335
Glu Glu Ile Glu Leu Gln Thr Ile Thr Pro Ser Thr Tyr Thr Thr Thr
340 345 350
Ser His Ala Ala Ser Pro Thr Ser Ile Asn Asn Gly Leu Tyr Asp Ile
355 360 365
Tyr Ala Asp Asp Phe Ile Thr Asp Thr Ser Thr Thr Pro Val Pro Ser
370 375 380
Val Pro Ser Thr Ser Leu Ser Gly Tyr Ile Pro Ala Asn Thr Thr Ile
385 390 395 400
Pro Phe Gly Gly Ala Tyr Asn Ile Pro Leu Val Ser Gly Pro Asp Ile
405 410 415
Pro Ile Asn Ile Thr Asp Gln Ala Pro Ser Leu Ile Pro Ile Val Pro
420 425 430
Gly Ser Pro Gln Tyr Thr Ile Ile Ala Asp Ala Gly Asp Phe Tyr Leu
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His Pro Ser Tyr Tyr Met Leu Arg Lys Arg Arg Lys Arg Leu Pro Tyr
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Phe Phe Ser Asp Val Ser
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<210> 8
<211> 488

<212> PRT
<213> Artificial Sequence

<220>
<223> HPV-16 L2/E7 fusion protein

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35 40 45
Gly Ser Met Gly Val Phe Phe Gly Gly Leu Gly Ile Gly Thr Gly Ser
50 55 60
Gly Thr Gly Gly Arg Thr Gly Tyr Ile Pro Leu Gly Thr Arg Pro Pro
65 70 75 80
Thr Ala Thr Asp Thr Leu Ala Pro Val Arg Pro Pro Leu Thr Val Asp
85 90 95
Pro Val Gly Pro Ser Asp Pro Ser Ile Val Ser Leu Val Glu Glu Thr
100 105 110
Ser Phe Ile Asp Ala Gly Ala Pro Thr Pro Val Pro Ser Ile Pro Pro
115 120 125
Asp Val Ser Gly Phe Ser Ile Thr Thr Ser Thr Asp Thr Thr Pro Ala
130 135 140
Ile Leu Asp Ile Asn Asn Thr Val Thr Thr Val Thr Thr His Asn Asn
145 150 155 160
Pro Thr Phe Thr Asp Pro Ser Val Leu Gln Pro Pro Thr Pro Ala Glu
165 170 175
Thr Gly Gly His Phe Thr Leu Ser Ser Ser Thr Ile Ser Thr His Asn
180 185 190
Tyr Glu Glu Ile Pro Met Asp Thr Phe Ile Val Ser Thr Asn Pro Asn
195 200 205
Thr Val Thr Ser Ser Thr Pro Ile Pro Gly Ser Arg Pro Val Ala Arg
210 215 220
Leu Gly Leu Tyr Ser Arg Thr Thr Gln Gln Val Lys Val Val Asp Pro
225 230 235 240
Ala Phe Val Thr Thr Pro Thr Lys Leu Ile Thr Tyr Asp Asn Pro Ala
245 250 255
Tyr Glu Gly Ile Asp Val Asp Asn Thr Leu Tyr Phe Pro Ser Asn Asp
260 265 270
Asn Ser Ile Asn Ile Ala Pro Asp Pro Asp Phe Leu Asp Ile Val Ala
275 280 285
Leu His Arg Pro Ala Leu Thr Ser Arg Arg Thr Gly Ile Arg Tyr Ser
290 295 300
Arg Ile Gly Asn Lys Gln Thr Leu Arg Thr Arg Ser Gly Lys Ser Ile
305 310 315 320
Gly Ala Lys Val His Tyr Tyr Tyr Asp Leu Ser Thr Ile Asn Pro Ala
325 330 335
Glu Glu Ile Glu Leu Gln Thr Ile Thr Pro Ser Thr Tyr Thr Thr Pro
340 345 350
Ser His Ala Ala Ser Pro Thr Ser Ile Asn Asn Gly Leu Tyr Asp Ile
355 360 365
Tyr Ala Asp Asp Phe Ile Thr Asp Thr Phe Thr Thr Pro Val Pro Ser
370 375 380
Ile Pro Ser Thr Ser Leu Ser Gly Tyr Ile Pro Ala Asn Thr Thr Ile
385 390 395 400
Pro Phe Gly Gly Ala Tyr Asn Ile Pro Leu Val Ser Gly Pro Asp Ile
405 410 415
Pro Ile Asn Thr Thr Asp Gln Thr Pro Ser Leu Ile Pro Ile Val Pro
420 425 430
Gly Ser Pro Gln Tyr Thr Ile Ile Ala Asp Gly Gly Asp Phe Tyr Leu
435 440 445
His Pro Ser Tyr Tyr Met Leu Arg Lys Arg Arg Lys Arg Leu Pro Tyr
450 455 460
Phe Phe Ser Asp Val Ser Met His Gly Asp Thr Pro Thr Leu His Glu
465 470 475 480
Tyr Met Leu Asp Leu Gln Pro Glu

485

<210> 9
<211> 805
<212> PRT
<213> Artificial Sequence

<220>
<223> HPV-16 L2/E7/E2 fusion protein

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35 40 45
Gly Ser Met Gly Val Phe Phe Gly Gly Leu Gly Ile Gly Thr Gly Ser
50 55 60
Gly Thr Gly Gly Arg Thr Gly Tyr Ile Pro Leu Gly Thr Arg Pro Pro
65 70 75 80
Thr Ala Thr Asp Thr Leu Ala Pro Val Arg Pro Pro Leu Thr Val Asp
85 90 95
Pro Val Gly Pro Ser Asp Pro Ser Ile Val Ser Leu Val Glu Glu Thr
100 105 110
Ser Phe Ile Asp Ala Gly Ala Pro Thr Ser Val Pro Ser Ile Pro Pro
115 120 125
Asp Val Ser Gly Phe Ser Ile Thr Thr Ser Thr Asp Thr Thr Pro Ala
130 135 140
Ile Leu Asp Ile Asn Asn Thr Val Thr Thr Val Thr Thr His Asn Asn
145 150 155 160
Pro Thr Phe Thr Asp Pro Ser Val Leu Gln Pro Pro Thr Pro Ala Glu
165 170 175
Thr Gly Gly His Phe Thr Leu Ser Ser Ser Thr Ile Ser Thr His Asn
180 185 190
Tyr Glu Glu Ile Pro Met Asp Thr Phe Ile Val Ser Thr Asn Pro Asn
195 200 205
Thr Val Thr Ser Ser Thr Pro Ile Pro Gly Ser Arg Pro Val Ala Arg
210 215 220
Leu Gly Leu Tyr Ser Arg Thr Thr Gln Gln Val Lys Val Val Asp Pro
225 230 235 240
Ala Phe Val Thr Thr Pro Thr Lys Leu Ile Thr Tyr Asp Asn Pro Ala
245 250 255
Tyr Glu Gly Ile Asp Val Asp Asn Thr Leu Tyr Phe Ser Ser Asn Asp
260 265 270
Asn Ser Ile Asn Ile Ala Pro Asp Pro Asp Phe Leu Asp Ile Val Ala
275 280 285
Leu His Arg Pro Ala Leu Thr Ser Arg Arg Thr Gly Ile Arg Tyr Ser
290 295 300
Arg Ile Gly Asn Lys Gln Thr Leu Arg Thr Arg Ser Gly Lys Ser Ile
305 310 315 320
Gly Ala Lys Val His Tyr Tyr Tyr Asp Phe Ser Thr Ile Asp Pro Ala
325 330 335
Glu Glu Ile Glu Leu Gln Thr Ile Thr Pro Ser Thr Tyr Thr Thr Thr
340 345 350
Ser His Ala Ala Ser Pro Thr Ser Ile Asn Asn Gly Leu Tyr Asp Ile
355 360 365
Tyr Ala Asp Asp Phe Ile Thr Asp Thr Ser Thr Thr Pro Val Pro Ser
370 375 380
Val Pro Ser Thr Ser Leu Ser Gly Tyr Ile Pro Ala Asn Thr Thr Ile
385 390 395 400
Pro Phe Gly Gly Ala Tyr Asn Ile Pro Leu Val Ser Gly Pro Asp Ile
405 410 415
Pro Ile Asn Ile Thr Asp Gln Ala Pro Ser Leu Ile Pro Ile Val Pro
420 425 430
Gly Ser Pro Gln Tyr Thr Ile Ile Ala Asp Ala Gly Asp Phe Tyr Leu
435 440 445

His Pro Ser Tyr Tyr Met Leu Arg Lys Arg Arg Lys Arg Leu Pro Tyr
450 455 460
Phe Phe Ser Asp Val Ser Met His Gly Asp Thr Pro Thr Leu His Glu
465 470 475 480
Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Gly Tyr Glu
485 490 495
Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala
500 505 510
Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys
515 520 525
Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr His Val
530 535 540
Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val
545 550 555 560
Cys Pro Ile Cys Ser Gln Lys Pro Ser Met Glu Thr Leu Cys Gln Arg
565 570 575
Leu Asn Val Cys Gln Asp Lys Ile Leu Thr His Tyr Glu Asn Asp Ser
580 585 590
Thr Asp Leu Arg Asp His Ile Asp Tyr Trp Lys His Met Arg Leu Glu
595 600 605
Cys Ala Ile Tyr Tyr Lys Ala Arg Glu Met Gly Phe Lys His Ile Asn
610 615 620
His Gln Val Val Pro Thr Leu Ala Val Ser Lys Asn Lys Ala Leu Gln
625 630 635 640
Ala Ile Glu Leu Gln Leu Thr Leu Glu Thr Ile Tyr Asn Ser Gln Tyr
645 650 655
Ser Asn Glu Lys Trp Thr Leu Gln Asp Val Ser Leu Glu Val Tyr Leu
660 665 670
Thr Ala Pro Thr Gly Cys Ile Lys Lys His Gly Tyr Thr Val Glu Val
675 680 685
Gln Phe Asp Gly Asp Ile Cys Asn Thr Met His Tyr Thr Asn Trp Thr
690 695 700
His Ile Tyr Ile Cys Glu Glu Ala Ser Val Thr Val Val Glu Gly Gln
705 710 715 720
Val Asp Tyr Tyr Gly Leu Tyr Tyr Val His Glu Gly Ile Arg Thr Tyr
725 730 735
Phe Val Gln Phe Lys Asp Asp Ala Glu Lys Tyr Ser Lys Asn Lys Val
740 745 750
Trp Glu Val His Ala Gly Gly Gln Val Ile Leu Cys Pro Thr Ser Val
755 760 765
Phe Ser Ser Asn Glu Val Ser Ser Pro Glu Ile Ile Arg Gln His Leu
770 775 780
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Glu Glu Thr Gln Thr
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<211> 536
<212> PRT
<213> Artificial Sequence

<220>
<223> HPV-16 L2/E6 fusion protein

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Ile Pro Lys Val Glu Gly Lys Thr Ile Ala Asp Gln Ile Leu Gln Tyr
35 40 45
Gly Ser Met Gly Val Phe Phe Gly Gly Leu Gly Ile Gly Thr Gly Ser
50 55 60
Gly Thr Gly Gly Arg Thr Gly Tyr Ile Pro Leu Gly Thr Arg Pro Pro
65 70 75 80
Thr Ala Thr Asp Thr Leu Ala Pro Val Arg Pro Pro Leu Thr Val Asp

85 90 95
Pro Val Gly Pro Ser Asp Pro Ser Ile Val Ser Leu Val Glu Glu Thr
100 105 110
Ser Phe Ile Asp Ala Gly Ala Pro Thr Ser Val Pro Ser Ile Pro Pro
115 120 125
Asp Val Ser Gly Phe Ser Ile Thr Thr Ser Thr Asp Thr Thr Pro Ala
130 135 140
Ile Leu Asp Ile Asn Asn Thr Val Thr Thr Val Thr Thr His Asn Asn
145 150 155 160
Pro Thr Phe Thr Asp Pro Ser Val Leu Gln Pro Pro Thr Pro Ala Glu
165 170 175
Thr Gly Gly His Phe Thr Leu Ser Ser Ser Thr Ile Ser Thr His Asn
180 185 190
Tyr Glu Glu Ile Pro Met Asp Thr Phe Ile Val Ser Thr Asn Pro Asn
195 200 205
Thr Val Thr Ser Ser Thr Pro Ile Pro Gly Ser Arg Pro Val Ala Arg
210 215 220
Leu Gly Leu Tyr Ser Arg Thr Thr Gln Gln Val Lys Val Val Asp Pro
225 230 235 240
Ala Phe Val Thr Thr Pro Thr Lys Leu Ile Thr Tyr Asp Asn Pro Ala
245 250 255
Tyr Glu Gly Ile Asp Val Asp Asn Thr Leu Tyr Phe Ser Ser Asn Asp
260 265 270
Asn Ser Ile Asn Ile Ala Pro Asp Pro Asp Phe Leu Asp Ile Val Ala
275 280 285
Leu His Arg Pro Ala Leu Thr Ser Arg Arg Thr Gly Ile Arg Tyr Ser
290 295 300
Arg Ile Gly Asn Lys Gln Thr Leu Arg Thr Arg Ser Gly Lys Ser Ile
305 310 315 320
Gly Ala Lys Val His Tyr Tyr Tyr Asp Phe Ser Thr Ile Asp Pro Ala
325 330 335
Glu Glu Ile Glu Leu Gln Thr Ile Thr Pro Ser Thr Tyr Thr Thr Thr
340 345 350
Ser His Ala Ala Ser Pro Thr Ser Ile Asn Asn Gly Leu Tyr Asp Ile
355 360 365
Tyr Ala Asp Asp Phe Ile Thr Asp Thr Ser Thr Thr Pro Val Pro Ser
370 375 380
Val Pro Ser Thr Ser Leu Ser Gly Tyr Ile Pro Ala Asn Thr Thr Ile
385 390 395 400
Pro Phe Gly Gly Ala Tyr Asn Ile Pro Leu Val Ser Gly Pro Asp Ile
405 410 415
Pro Ile Asn Ile Thr Asp Gln Ala Pro Ser Leu Ile Pro Ile Val Pro
420 425 430
Gly Ser Pro Gln Tyr Thr Ile Ile Ala Asp Ala Gly Asp Phe Tyr Leu
435 440 445
His Pro Ser Tyr Tyr Met Leu Arg Lys Arg Arg Lys Arg Leu Pro Tyr
450 455 460
Phe Phe Ser Asp Val Ser Met His Gln Lys Arg Thr Ala Met Phe Gln
465 470 475 480
Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu Cys Thr Glu Leu
485 490 495
Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val Tyr Cys Lys Gln
500 505 510
Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe Arg Asp Leu Cys
515 520 525
Ile Val Tyr Arg Asp Gly Asn Pro
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<211> 1518
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L1 wild type NVAX clone

<400> 11

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cagtttcctt taggacgcaa atttttacta caagcaggat tgaaggccaa accaaaattt 1440
acattaggaa aacgaaaagc tacacccacc acctcatcta cctctacaac tgctaaacgc 1500
aaaaaacgta agctgtaa 1518

<210> 12
<211> 1518
<212> DNA
<213> Artificial Sequence

<220>
<223> HPV-16 L1 wild type clone (GenBank K02718)

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agactacttg cagttggaca tccctatttt cctattaaaa aacctaacaa taacaaaata 180
ttagttccta aagtatcagg attacaatac agggtattta gaatacattt acctgacccc 240
aataagtttg gttttcctga cacctcattt tataatccag atacacagcg gctggtttgg 300
gcctgtgtag gtgttgaggt aggtcgtggt cagccattag gtgtgggcat tagtggccat 360
cctttattaa ataaattgga tgacacagaa aatgctagtg cttatgcagc aaatgcaggt 420
gtggataata gagaatgtat atctatggat tacaaacaaa cacaattgtg tttaattggt 480
tgcaaaccac ctatagggga acactggggc aaaggatccc catgtaccaa tgttgcagta 540
aatccaggtg attgtccacc attagagtta ataaacacag ttattcagga tggtgatatg 600
gttcatactg gctttggtgc tatggacttt actacattac aggctaacaa aagtgaagtt 660
ccactggata tttgtacatc tatttgcaaa tatccagatt atattaaaat ggtgtcagaa 720
ccatatggcg acagcttatt tttttattta cgaagggaac aaatgtttgt tagacattta 780
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